This is a digital copy of a book that was preserved for generations on library shelves before it was carefully scanned by Google as part of a project to make the world's books discoverable online.

It has survived long enough for the copyright to expire and the book to enter the public domain. A public domain book is one that was never subject to copyright or whose legal copyright term has expired. Whether a book is in the public domain may vary country to country. Public domain books are our gateways to the past, representing a wealth of history, culture and knowledge that's often difficult to discover.

Marks, notations and other marginalia present in the original volume will appear in this file - a reminder of this book's long journey from the publisher to a library and finally to you.

Usage guidelines

Google is proud to partner with libraries to digitize public domain materials and make them widely accessible. Public domain books belong to the public and we are merely their custodians. Nevertheless, this work is expensive, so in order to keep providing this resource, we have taken steps to prevent abuse by commercial parties, including placing technical restrictions on automated querying.

We also ask that you:

+ *Make non-commercial use of the files* We designed Google Book Search for use by individuals, and we request that you use these files for personal, non-commercial purposes.
+ *Refrain from automated querying* Do not send automated queries of any sort to Google's system: If you are conducting research on machine translation, optical character recognition or other areas where access to a large amount of text is helpful, please contact us. We encourage the use of public domain materials for these purposes and may be able to help.
+ *Maintain attribution* The Google "watermark" you see on each file is essential for informing people about this project and helping them find additional materials through Google Book Search. Please do not remove it.
+ *Keep it legal* Whatever your use, remember that you are responsible for ensuring that what you are doing is legal. Do not assume that just because we believe a book is in the public domain for users in the United States, that the work is also in the public domain for users in other countries. Whether a book is still in copyright varies from country to country, and we can't offer guidance on whether any specific use of any specific book is allowed. Please do not assume that a book's appearance in Google Book Search means it can be used in any manner anywhere in the world. Copyright infringement liability can be quite severe.

About Google Book Search

Google's mission is to organize the world's information and to make it universally accessible and useful. Google Book Search helps readers discover the world's books while helping authors and publishers reach new audiences. You can search through the full text of this book on the web at `http://books.google.com/`

HARVARD MEDICAL LIBRARY
IN THE
FRANCIS A. COUNTWAY
LIBRARY OF MEDICINE

THE JOURNAL OF
EXPERIMENTAL MEDICINE

THE JOURNAL
OF
EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

VOLUME THIRTY-FIFTH

WITH SIXTY-SEVEN PLATES AND
ONE HUNDRED AND THIRTY FIGURES
IN THE TEXT

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1922

LIBRARY OF THE HARVARD MEDICAL SCHOOL

41

COPYRIGHT, 1922, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

CONTENTS.

No. 1, January 1, 1922.

PAGE

Olitsky, Peter K., and Gates, Frederick L. Experimental studies of the nasopharyngeal secretions from influenza patients. VI. Immunity reactions........................ 1

Stillman, Ernest G. The frequency of *Bacillus influenzæ* in the nose and throat in acute lobar pneumonia.............. 7

Carrel, Alexis, and Ebeling, Albert H. Heterogenic serum, age, and multiplication of fibroblasts.................... 17

Pearce, Louise, and Brown, Wade H. A study of the relation of *Treponema pallidum* to lymphoid tissues in experimental syphilis... 39

Gates, Frederick L. Studies on agglutination with the aid of the centrifuge. The influence of temperature on absorption and flocculation.................................. 63

Drinker, Cecil K., Peabody, Francis W., and Blumgart, Herrmann L. The effect of pulmonary congestion on the ventilation of the lungs. Plates 1 to 3................... 77

No. 2, February 1, 1922.

Auer, John. Experimental generalized analgesia after exposure to some war gases. Plates 4 to 6........................ 97

Olitsky, Peter K. Experimental studies on the etiology of typhus fever. II. Survival of the virus in aerobic and anaerobic culture media................................ 115

Olitsky, Peter K. Experimental studies on the etiology of typhus fever. III. Filtration experiments................ 121

McMaster, Philip D. Do species lacking a gall bladder possess its functional equivalent?........................ 127

Robertson, Oswald H., and Rous, Peyton. Sources of the antibodies developing after repeated transfusion........... 141

NYE, ROBERT N. Studies on the pneumonic exudate. V. The relation of pneumonic lung protease activity to hydrogen ion concentration, and a consideration of the origin of the enzyme.......... 153
LITTLE, RALPH B., and ORCUTT, MARION L. The transmission of agglutinins of *Bacillus abortus* from cow to calf in the colostrum.......... 161
HAMMETT, FREDERICK S., NOWREY, JOSEPH E., JR., and MÜLLER, JOHN H. The erythropoietic action of germanium dioxide.. 173
CHESNEY, ALAN M. The use of phenol red and brom-cresol purple as indicators in the bacteriological examination of stools.......... 181
WARREN, S. L., and WHIPPLE, G. H. Roentgen ray intoxication. I. Unit dose over thorax negative—over abdomen lethal. Epithelium of small intestine sensitive to x-rays.......... 187
WARREN, S. L., and WHIPPLE, G. H. Roentgen ray intoxication. II. A study of the sequence of clinical, anatomical, and histological changes following a unit dose of x-rays.......... 203
WARREN, S. L., and WHIPPLE, G. H. Roentgen ray intoxication. III. Speed of autolysis of various body tissues after lethal x-ray exposures. The remarkable disturbance in the epithelium of the small intestine.......... 213
MCQUARRIE, IRVINE, and WHIPPLE, G. H. A study of renal function in Roentgen ray intoxication. Resistance of renal epithelium to direct radiation.......... 225
CUTLER, ELLIOTT C. The relation of the hypophysis to antibody production.......... 243
DUVAL, CHARLES W., and D'AUNOY, RIGNEY. Studies upon experimental measles. I. The effects of the virus of measles upon the guinea pig.......... 257
FENN, WALLACE O. Hemolysis of erythrocytes in contact with glass.......... 271

NO. 3, MARCH 1, 1922.

GRATIA, ANDRÉ. The Twort-d'Hérelle phenomenon. II. Lysis and microbic variation. Plates 7 and 8.......... 287

KLIGLER, I. J., and ROBERTSON, O. H. The cultivation and biological characteristics of *Spirochæta obermeieri* (*recurrentis*) 303

LEWIS, MARGARET REED. The importance of dextrose in the medium of tissue cultures.................................. 317

STADIE, WILLIAM C. Construction of an oxygen chamber for the treatment of pneumonia. Plate 9.................... 323

STADIE, WILLIAM C. The treatment of anoxemia in pneumonia in an oxygen chamber.................................... 337

JONES, F. S. An organism resembling *Bacillus actinoides* isolated from pneumonic lungs of white rats. Plate 10............ 361

FISCHER, ALBERT. A three months old strain of epithelium. Plates 11 to 14.. 367

MURPHY, JAMES B., LIU, J. HENG, and STURM, ERNEST. Studies on x-ray effects. IX. The action of serum from x-rayed animals on lymphoid cells *in vitro*. Plate 15.............. 373

CLARK, HARRY. The measurement of intravenous temperatures. Plate 16... 385

NOGUCHI, HIDEYO. Venereal spirochetosis in American rabbits. Plates 17 to 20.. 391

No. 4, APRIL 1, 1922.

ORCUTT, MARION L., and HOWE, PAUL E. Hemolytic action of a staphylococcus due to a fat-splitting enzyme............. 409

PAPPENHEIMER, A. M., MCCANN, G. F., and ZUCKER, T. F. Experimental rickets in rats. IV. The effect of varying the inorganic constituents of a rickets-producing diet. Plates 21 to 24.. 421

PAPPENHEIMER, A. M., MCCANN, G. F., and ZUCKER, T. F. Experimental rickets in rats. V. The effect of varying the organic constituents of a rickets-producing diet. Plates 25 to 29.. 447

BROWN, J. HOWARD. Modification of an improved anaerobe jar.. 467

OLITSKY, PETER K. Experimental studies on the etiology of typhus fever. IV. Immunizing and toxic agents found occasionally in filtrates of typhus-infected tissues. Plates 30 and 31.. 469

NAKAHARA, WARO, and MURPHY, JAMES B. Studies on x-ray effects. X. The biological action of small doses of low frequency x-rays. Plates 32 and 33 475

LIU, J. HENG, STURM, ERNEST, and MURPHY, JAMES B. Studies on x-ray effects. XI. The fate of cancer grafts implanted in subcutaneous tissue previously exposed to x-rays 487

NAKAHARA, WARO. Studies on lymphoid activity. VI. Immunity to transplanted cancer induced by injection of olive oil. Plates 34 to 36 493

HAMMETT, FREDERICK S., and NOWREY, JOSEPH E., JR. The erythropoietic action of germanium dioxide. II. The source of the erythrocythemia produced by germanium dioxide in the albino rat 507

HAESSLER, HERBERT. The effect of flood diuresis on hemoglobinuria 515

McMASTER, PHILIP D., ROUS, PEYTON, and LARIMORE, LOUISE C. Significance of the hemosiderosis of pernicious anemia 521

HAESSLER, HERBERT, ROUS, PEYTON, and BROUN, G. O. The renal elimination of bilirubin 533

OLITSKY, PETER K., and GATES, FREDERICK L. Experimental studies of the nasopharyngeal secretions from influenza patients. VII. Serological reactions. Plate 37 553

DE KRUIF, PAUL H. Mutation of the bacillus of rabbit septicemia. Plate 38 561

DU NOÜY, P. LECOMTE. Spontaneous decrease of the surface tension of serum. I 575

No. 5, MAY 1, 1922.

SWIFT, HOMER F., BOOTS, RALPH H., and MILLER, C. PHILIP, JR. A cutaneous nematode infection in monkeys. Plates 39 to 52 599

DE KRUIF, PAUL H. Virulence and mutation of the bacillus of rabbit septicemia 621

GATES, FREDERICK L. Collodion sacs for aerobic and anaerobic bacterial cultivation. Plates 53 and 54 635

CARREL, ALEXIS, and EBELING, ALBERT H. Heat and growth-inhibiting action of serum 647

EBELING, ALBERT H. Cicatrization of wounds. XIII. The temperature coefficient.................................. 657
FISCHER, ALBERT. Action of antigen on fibroblasts *in vitro* 661
BROWN, J. HOWARD. The vaseline tube and syringe method of micro gas analysis of bacterial cultures. Plates 55 to 57... 667
LORD, FREDERICK T., and NYE, ROBERT N. Studies on the pneumococcus. I. Acid death-point of the pneumococcus.. 685
LORD, FREDERICK T., and NYE, ROBERT N. Studies on the pneumococcus. II. Dissolution of pneumococci at varying hydrogen ion concentrations. Effect of temperature, previous killing of the organisms, and fresh human serum on the phenomenon. Behavior of other organisms......... 689
LORD, FREDERICK T., and NYE, ROBERT N. Studies on the pneumococcus. III. Dissolution of pneumococci in pneumonic cellular material at varying hydrogen ion concentrations. Resistance of certain other organisms to dissolution.. 699
LORD, FREDERICK T., and NYE, ROBERT N. Studies on the pneumococcus. IV. Effect of bile at varying hydrogen ion concentrations on dissolution of pneumococci.............. 703
DU NOÜY, P. LECOMTE. Surface tension of serum. II. Action of time on the surface tension of serum solutions. Plates 58 to 61... 707
FOOT, NATHAN CHANDLER. Pathology of the dermatitis caused by *Megalopyge opercularis*, a Texan caterpillar. Plates 62 and 63... 737

No. 6, JUNE 1, 1922.

EBELING, ALBERT H. A ten year old strain of fibroblasts. Plates 64 to 66.. 755
FELTON, LLOYD D., and DOUGHERTY, KATHARINE M. Study of the action of four aromatic cinchona derivatives on pneumococcus. A comparison with optochin................. 761
TYZZER, ERNEST EDWARD, and FABYAN, MARSHAL. A further inquiry into the source of the virus in blackhead of turkeys, together with observations on the administration of ipecac and of sulfur.. 791

OLITSKY, PETER K., and GATES, FREDERICK L. Experimental studies of the nasopharyngeal secretions from influenza patients. VIII. Further observations on the cultural and morphological characters of *Bacterium pneumosintes*. Plate 67 813
STEVENS, FRANKLIN A., and WEST, RANDOLPH. The peptase, lipase, and invertase of hemolytic streptococcus 823
KRUMBHAAR, EDWARD B., and CHANUTIN, ALFRED. Studies on experimental plethora in dogs and rabbits 847
INDEX TO VOLUME XXXV 873

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

VI. IMMUNITY REACTIONS.

BY PETER K. OLITSKY, M.D., AND FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 16, 1921.)

In the earlier experiments of this series in which the anaerobic, filter-passing microorganism, *Bacterium pneumosintes*, was transmitted from the nasopharyngeal secretions of influenza patients to the respiratory tract of rabbits, and passed from rabbit to rabbit through many generations, it was our practice to inoculate two or more rabbits in each experiment. One or more of these animals was killed after 24 to 48 hours, as already described,[1] for study and to obtain material for further inoculation. The infection, however, was permitted to run its course in a number of rabbits, which upon recovery became available for subsequent studies in immunity.

The experiments made with the latter rabbits may be grouped according to the nature of the injected material (Table I). These animals were reinjected after varying intervals with material from other sources. The tests were particularly useful in establishing the identity of the active agent derived from human nasopharyngeal washings, and from the lung tissues of affected rabbits, with the cultures of *Bacterium pneumosintes*.

A single typical protocol of the experiments will serve to show the method followed and the results obtained.

Group A. Influenzal Nasopharyngeal Washings versus Active Agent in Lungs of Affected Rabbits.

Experiment 1.—Rabbit A for 4 days prior to injection showed an average leucocyte count of 11,821, of which 6,190 were mononuclears, and temperature of 39.6°C. Mar. 29, 1919. Inoculated intratracheally with 3 cc. of the unfiltered nasopharyngeal washings from Case 16.[1] Mar. 30. Conjunctivitis. Total leucocytes 9,000, of which 3,600 were mononuclears. Temperature 39.6°C. Mar. 31. Conjunctivitis. Total leucocytes 7,025, of which 1,967 were mononuclears. Temperature 40°C. Apr. 1. Conjunctivitis. Total leucocytes 6,325, of which 2,404 were mononuclears. Temperature 40°C. Apr. 2. Recovered. Total leucocytes 11,500, of which 4,140 were mononuclears. Temperature 39.4°C. The animal remained well until Apr. 21, 1919, when it was reinjected, intratracheally, with 3 cc. of lung tissue suspension from a rabbit representing the fifth passage of the nasopharyngeal secretions from Case 17.[1] This active material had been filtered between the third and fourth rabbit passages. No observable effects on the rabbit's appearance, behavior, blood count, or temperature resulted.

---

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

As a control for the first injection, Rabbit B was injected along with Rabbit A, and with the same material. For 2 days after the injection Rabbit B showed a typical clinical picture, with a fall in the leucocyte count, affecting especially the mononuclears, and a rise in temperature. The animal was then killed and autopsy revealed the characteristic hemorrhagic edema and emphysema of the lungs, without involvement of other organs.[1] Similarly, Rabbit C served as a control for the second injection of Rabbit A, and showed typical clinical and pathological effects.

TABLE I.

Groups of Immunity Experiments.

Group.	First injection.	Second injection.
A	Nasopharyngeal washings from influenza patients.	Suspensions of lungs of affected rabbits.
B	Suspensions of lungs of affected rabbits.	Similar suspensions from another animal.
C	Washed mass cultures of *Bacterium pneumosintes*.	Similar cultures of another strain.
D	Suspensions of lungs of affected rabbits.	Washed mass cultures of *Bacterium pneumosintes*.
E	Washed mass cultures of *Bacterium pneumosintes*.	Suspensions of lungs of affected rabbits.

This experiment, typical of three in this series, indicates the development of a protective resistance to the active agent in the rabbit passage experiments, as the result of the previous injection of the nasopharyngeal secretions from an early case of influenza. The nasopharyngeal secretions are thus further shown to be the source of the filterable active material of the rabbit passages. In this instance, the interval between injections was 23 days. In another experiment, the resistance was maintained for 3 months.

Group B. Active Agent in Lungs of Affected Rabbits versus Similar Material Originally Derived from Other Patients.

That the strains of the active agent in lungs of affected rabbits, originally derived from different influenza patients, have the same antigenic properties and thus give rise to protection against each other is brought out by the following experiment.

Experiment 2.—Rabbit A, having a normal leucocyte count of 14,525, of which 6,100 were mononuclears, and a temperature of 39.8°C., was injected intratracheally on Feb. 25, 1919, with 3 cc. of a saline solution suspension of rabbit lung from the fourth passage of the nasopharyngeal secretions of Case 11,[1] obtained during the first influenzal wave of 1918–19. The subsequent train of events carried the mononuclear cells to 1,242, and the temperature to 40.65°C. on the 3rd day. The fever and mononuclear leucopenia persisted on the 4th and 5th days, with recovery on the 6th and 7th days, when the total leucocyte count was 20,450, of which 5,521 were mononuclears, and temperature 39.6°C. Nearly 14 months later, May 11, 1920, this animal was injected with 3 cc. of a suspension of glycerolated lung tissue from the first rabbit inoculated with the nasopharyngeal secretions of Case 26,[1] from the second or 1920 epidemic. The rabbit developed no significant changes in the blood count or temperature, and when killed, on May 13, showed no visible lung lesions.

The control rabbit, B, similarly injected with the same glycerolated lung tissue, developed the typical blood picture and lung lesions, and the presence of the active agent in the lungs was demonstrated by transmission to a second rabbit which reacted in the characteristic manner.

This experiment not only demonstrates the identity of the antigenic characters of the active material derived from the two epidemics of 1918–19 and of 1920, but shows that the immunity conferred by a previous infection with the active agent may last for a long time.

With the isolation of *Bacterium pneumosintes* from the nasopharyngeal secretions of influenza patients and from the lung tissue of affected rabbits, it became possible to study the character of the active agent of the animal passages by means of cross-protection experiments. It was first necessary to show that recovery from infection with *Bacterium pneumosintes* induces resistance to a subsequent inoculation of the same microorganism.

Group C. Bacterium pneumosintes versus the Same Microorganism.

Experiment 3.—Rabbit A, normal leucocyte count 9,600, of which 6,144 were mononuclears. Temperature 39.3°C. Injected intratracheally with 3 cc. of a saline suspension of washed mass culture in the seventh generation of a rabbit lung strain of *Bacterium pneumosintes* from Case 16,[1] a patient from the first (1918–19) epidemic wave. 1st day after injection: total leucocytes 8,400, of which 2,520 were mononuclears; temperature 39.3°C. 2nd day: total leucocytes 3,800, of which 1,444 were mononuclears; temperature 39.6°C. 3rd day: total leucocytes 5,400, of which 2,052 were mononuclears; temperature 39.6°C. 4th day: recovery; total leucocytes 11,600, of which 4,640 were mononuclears; temperature 39.3°C. 5th day: total leucocytes 15,000, of which 7,500 were mononuclears; temperature 39.4°C. On the following day, when the total leucocytes were 13,600, of which 5,440 were mononuclears, and the temperature was 39.8°C., this animal was reinjected with a similar washed mass culture of a strain of *Bacterium pneumosintes* in the seventh generation from the filtered nasopharyngeal secretions of Case 17,[1] from the same epidemic. During the next 5 days the total leucocyte count remained about 13,000, with an increase in the mononuclear cells to an average of 8,292, and the temperature was constant at 39.7°C.

For assurance that the injected mass culture was active and capable of infecting normal rabbits, a control animal was injected, each time, along with Rabbit A, and with the same material. For 48 hours after injection, these rabbits, B and C, showed the characteristic leucocytic depression, involving especially the mononuclears. In each instance they were then killed, and the usual emphysema and hemorrhagic edema of the lungs were found.[2]

To show that the reactions described were due to *Bacterium pneumosintes* and not to some constituent of the culture medium, Rabbit D was inoculated intratracheally with 3 cc. of a saline suspension of the sediment of uninoculated, sterile, mass culture medium, at the time that Rabbit A was first injected. The animal gave no reaction. At the time of the second injection, Rabbit D was also injected with the seventeenth generation of the culture of the nasopharyngeal secretions of Case 17. This animal promptly developed the clinical and pathological syndrome characteristic of infection with *Bacterium pneumosintes*.[2]

Group D. Active Agent in Lungs of Affected Rabbits versus Bacterium pneumosintes.

We have already stated[2] that "the active material, pathogenic for rabbits and guinea pigs, found in the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza has been identified in the anaerobic organism" (*Bacterium pneumosintes*).

[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

Additional evidence in support of this statement is furnished by the results of cross-protection experiments of which the following are examples.

Experiment 4.—Rabbit A, normal leucocyte count, 9,200, of which 6,072 were mononuclears. Temperature 39°C. Injected intratracheally with 3 cc. of a saline solution suspension of rabbit lung tissue representing the fifth passage of the filtered nasopharyngeal secretions from Case 26.[1] The following day, leucocytes were 6,400, of which 2,560 were mononuclears. Temperature 39.3°C. 2nd day: leucocytes 7,200, of which 2,763 were mononuclears; temperature 39.4°C. 3rd day: leucocytes 4,400, of which 1,760 were mononuclears; temperature 39.4°C. 4th day: recovery with leucocytes 11,000, of which 6,600 were mononuclears; temperature 39.2°C. 15 days later this rabbit was injected intratracheally with 3 cc. of a saline solution suspension of washed mass culture of *Bacterium pneumosintes*, second generation, originally derived from the nasopharyngeal secretions of the same patient. On the 2 days following, the total leucocyte counts were 14,600 and 16,800, of which 7,800 and 9,240 were mononuclears; temperature 39.5°C. Killed. Autopsy revealed no visible lesions in the lungs. The activity of the materials used in these injections was demonstrated by the injection of control rabbits, B and C, both of which gave typical reactions. In similar experiments, the reinjected rabbits were observed for several days after the second injection to detect any delayed effects. They remained normal.

In Experiment 4, the interval between the first and second injections was 15 days. In three other experiments, identical results were obtained after 3 weeks, and in a fifth, after 9 weeks. In a sixth experiment, involving an interval of 18 days, the second injection produced the typical clinical and pathological reaction. That such protection experiments should occasionally be unsuccessful is not surprising, in view of the arbitrary dosage employed.

Group E. Bacterium pneumosintes versus Active Agent in Lungs of Affected Rabbits.

The converse of Experiment 4 was performed in a series in which *Bacterium pneumosintes* was injected first and the animals were later exposed to the influenzal active agent by the intratracheal injection of affected rabbit lung.

Experiment 5.—Rabbit A showed a normal leucocyte count of 12,000, of which 6,240 were mononuclears. Temperature 39°C. It was injected intratracheally with 3 cc. of a saline solution suspension of washed sediment from a mass culture of *Bacterium pneumosintes.* During the following 3 days, the total leucocyte count averaged 7,200, of which 2,200 were mononuclears, and the temperature rose to 39.6°C. Thereafter the animal returned to normal. 2 weeks after the first injection, this rabbit was inoculated intratracheally with 3 cc. of a suspension of the active agent in rabbit lung tissue from the fifth passage of the nasopharyngeal secretions of Case 26.[1] After waiting for 48 hours, during which time no change occurred in the blood or temperature, the rabbit was killed. The lungs appeared normal.

In three other similar experiments the rabbits were observed for several days after the second injection, and remained normal. The interval between injections in this series was 11 days, 2, 7, and 8 weeks. The usual control animals, B and C, demonstrated the activity of the injected materials in each test.

These cross-protection experiments establish the antigenic identity of *Bacterium pneumosintes* with the active agent derived from the nasopharyngeal secretions of patients in the early hours of epidemic influenza.

SUMMARY AND CONCLUSION.

The experiments described furnish additional evidence of the pathogenic character and the virtual identity of the various strains of the active agent derived from the nasopharyngeal secretions of influenzal patients with which the transmission experiments in rabbits have been carried out.

The active material has been shown to be of antigenic nature, so that rabbits are protected from the effects of a second inoculation. The experiments indicate also the antigenic identity of the various strains of the active agent with each other and with *Bacterium pneumosintes.*

Finally, the experiments show that the protection may persist for 14 months which is the longest period yet tested.

THE FREQUENCY OF BACILLUS INFLUENZÆ IN THE NOSE AND THROAT IN ACUTE LOBAR PNEUMONIA.

BY ERNEST G. STILLMAN, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, June 15, 1921.)

The writer has for some time been engaged in the study of the flora of the nose and throat both in well persons and in those suffering from infections of the respiratory tract. Especial attention has been given to the presence of *Bacillus influenzæ* and pneumococcus, and some of the results obtained have already been published (1).

During the course of these studies, certain observations have been made concerning the frequency of occurrence of influenza bacilli and pneumococci in the nose and throat, and concerning the varieties of influenza bacilli which are present in these regions. It is possible that these findings may have a bearing upon the etiology of lobar pneumonia, and for this reason they are reported here.

Methods.

The throat cultures were made by means of cotton swabs passed over the posterior wall of the pharynx. Two swabs were used in each case—with one a freshly poured oleate hemoglobin agar plate was inoculated, with the other a fresh blood agar plate.

Cultures from the nose were made by means of a cotton swab passed through a sterile speculum in order to avoid contamination from the nasal hairs. Cultures were usually made from the nasal mucous membrane in the region of the middle turbinate, though occasionally a deviated septum prevented the introduction of the swab as far as this region. The swabs were streaked over freshly poured blood agar plates.

Attention was given not only to the occurrence of colonies of pneumococcus and of *Bacillus influenzæ* on the various plates, but an effort was made to determine the type of influenza bacilli present and especially whether bacilli of more than one type could be isolated from a single culture. For this reason, whenever colonies of *Bacillus influenzæ* were present, several individual colonies were marked and a secondary culture on a fresh blood agar slant was made from each of the colonies. In the present paper, the occurrence of hemoglobinophilic bacilli producing hemolysis on blood agar plates, the so called Bacillus X, is not discussed and the presence of this organism is not noted. All organisms here described as *Bacillus influenzæ* failed to produce hemolysis.

The media used in this study had an initial hydrogen ion concentration of about pH 7.4. The blood agar was prepared by adding sterile defibrinated rabbit blood to melted agar to a concentration of 4 per cent or by adding blood extract, according to Wollstein's (2) method, to a concentration of 2 per cent.

In this connection, it should be mentioned that all solid media employed in the cultivation of *Bacillus influenzæ* should be freshly prepared. The addition of a little broth to the surface of old blood agar plates will not give satisfactory results. In the present study, the plates were poured within an hour of use. For making fresh oleate hemoglobin plates, it is well to employ tubes of oleate medium which may be melted as needed. Care must be taken that the blood cells are completely hemolyzed before the oleate agar is tubed. If this is not done, upon remelting the agar a heavy protein precipitate will be formed.

Indole production was tested by Ehrlich's para-dimethylaminobenzaldehyde method. The clear supernatant fluid from 48 hour cultures in blood broth or in Dunham's peptone solution enriched with blood extract was used. Since an occasional strain, which apparently had grown luxuriantly, failed to produce indole on the first test, all tests resulting negatively were repeated at least once.

Gas production was tested by making either stab or shake cultures in 1 per cent dextrose agar to which blood extract had been added. Since the amount of gas produced is never very great, all negative tests were repeated.

The sugar fermentation tests were made with cultures in Dunham's peptone solution enriched with 2 per cent blood extract. All culture tubes were incubated in a slanted position so as to expose as large a surface of the medium as possible to the air, since acid production has been shown to be more rapid under these conditions. As a routine procedure, dextrose, which 92 per cent of the strains fermented, was used as a control, and saccharose as the differential sugar.

Occurrence of Bacillus influenzæ in the Upper Respiratory Tract of Normal Individuals and of Individuals Suffering from Lobar Pneumonia.

During the recent epidemic of influenza in New York in 1918–19 and in the succeeding winters, the writer made many cultures from the throats of normal individuals to determine the presence or absence of *Bacillus influenzæ*. During the period 1918–21, cultures were made from the throats of 1,077 normal individuals, and influenza bacilli were demonstrated to be present in 332, or in 30 per cent (Table I).

While cultures have been made from the nose in a much smaller number of normal individuals and then only during the past year, 1920–21, the results indicate that hemoglobinophilic bacilli are rarely found here, even in persons suffering from coryza, laryngitis, etc. (Table II).

In marked contrast with these findings are the results obtained in a series of cultures made from the nose and throat of patients suffering from lobar pneumonia. In thirty-one of these cases, cultures were made from the throat, and in eighteen, or from 58 per cent of the cases, influenza bacilli were isolated (Table I). In thirty-five cases of pneumonia, cultures were also made from the nose and influenza bacilli were isolated from this source in nine, or in 23 per cent (Table II). In certain other cases influenza bacilli were isolated from the sputum when they could not be demonstrated in the cultures from the nose or throat. Among the entire thirty-five cases, influenza bacilli were isolated from at least one of these three sources in thirty, or 85 per cent of the cases. These cultures were made during the winter of 1920–21, 2 years after the severe epidemic of influenza. During this winter few cases of typical influenza occurred in New York. That this great frequency of association of *Bacillus influenzæ* with patients suffering from pneumonia is not directly related to the epidemic is further rendered probable by the fact that during the 5 years previous to the epidemic, namely from 1913–18, influenza bacilli were isolated from the sputum of 20 per cent of 672 cases of pneumonia studied in the Hospital of The Rockefeller Institute. While the relative number of instances in which influenza bacilli were isolated from this series of pneumonia patients is not as great as the percentage of normal persons who in 1918–21 were found to be carrying these organisms, it must be borne in mind that influenza bacilli were only incidentally looked for in the pneumonia patients previous to 1918 and were only brought to our attention by their growth in the mouse into which sputum had been injected. These figures indicate that influenza bacilli

TABLE I.

Incidence of B. influenzæ in Throat Cultures from Normal Individuals and from Individuals Suffering from Lobar Pneumonia.

Date.	Total No. of individuals.	Positive cultures.	Per cent positive.
Normal individuals.			
Winter of 1918–19	717	256	35
" " 1919–20	253	44	17
" " 1920–21	107	32	29
Total	1,077	332	30
Patients with lobar pneumonia.			
1920–21	31	18	58

TABLE II.

Incidence of B. influenzæ in the Nose of Normal Individuals and of Individuals Suffering from Acute Coryza, Laryngitis, Encephalitis Lethargica, and Lobar Pneumonia.

Source of culture.	No. of cases.	Cultures.	*B. influenzæ* present.
Normal individuals	22	28	–
Acute coryza and laryngitis	13	23	–
Encephalitis lethargica	2	2	–
Lobar pneumonia	35	35	9

are much more frequently present in the upper respiratory tract of patients with lobar pneumonia than of normal persons, or at any rate can be more frequently isolated. This is especially true of the occurrence of these bacilli in the throat but is also true of their occurrence in the nose.

In searching for influenza bacilli in the nose, we have also noted the presence or absence of pneumococcus. In the cultures from the nose of the twenty-two normal individuals mentioned in Table II, it was possible to isolate pneumococcus in only one instance. More surprising were the results obtained from nasal cultures from the thirteen cases of coryza and laryngitis and from the two cases of encephalitis lethargica. In these instances no pneumococci were found. Park (3), Allen (4), and others have previously shown that cultures from the nose of normal individuals usually yield a very scanty growth of bacteria. Not infrequently cultures from this region have been found to be sterile.

On the other hand, we have made cultures from the nose in thirty-five cases of lobar pneumonia, and in fifteen instances pneumococci were demonstrated to be present. In the majority of instances, the type of pneumococcus isolated was determined and the organism was found to be identical with those associated with the lung lesion as shown by the sputum determination.

Types of Bacillus influenzæ in Nose, Sputum, and Throat Cultures from Individuals Suffering from Lobar Pneumonia.

In the classification of the influenza bacilli, use was made of the grouping devised by Stillman and Bourn (5). The following chart indicates briefly the method employed and the mode of identifying the various groups.

	Group.					
	A.	B.	C.	D.	E.	F.
Indole	+	−	−	−	+	−
Gas	−	+	−	−	−	+
Saccharose	−	+	+	−	+	−

From a study of the hemoglobinophilic bacilli isolated, it soon became evident that the organisms present in any one case or even in any one culture were not all of the same type. It was therefore impossible to group the cases according to the presence of *Bacillus* *influenzæ* of the different types or even of the prevailing type. A study was made, therefore, of all the strains isolated from cases of lobar pneumonia during the winter of 1920–21 and the results are shown in Table III.

It is evident from this table that the strains of *Bacillus influenzæ* isolated from the nose, throat, and sputum of patients with lobar pneumonia do not belong in any one group, but that representatives of all groups occur, though the strains belonging in Group A are in the majority.

Of still greater importance than this, however, is the fact that in certain instances the study of several colonies from the same culture plate from the nose or throat has indicated that influenza bacilli of several different groups may be simultaneously present. For instance, in one case from a single plate made from the throat, of five colonies of *Bacillus influenzæ* studied, two colonies were shown to consist of influenza bacilli of Group C, and three of influenza bacilli of Group D, and in another case three colonies from a throat culture plate were studied and the bacilli from each of the colonies were found to belong in a different group.

TABLE III.

A Biological Classification of 140 Strains of B. influenzæ Recovered from the Throat, Sputum, and Nose of Thirty-five Individuals Suffering from Lobar Pneumonia.

Source of culture.	Total No. of cultures.	No. of strains studied.	Group.					
			A.	B.	C.	D.	E.	F.
Throat........................	32	91	32	18	7	32	2	—
Sputum *via* mouse...............	8	18	8	—	2	8	—	—
Nasal..........................	35	31	18	2	1	10	—	—
Total.......................	75	140	58	20	10	50	2	—

B. influenzæ present in 30 cases.
" " absent " 5 "
Total 35 "

Of seven cases of lobar pneumonia in which both nose and throat cultures yielded *Bacillus influenzæ*, in three a single type of organism was recovered from both nose and throat. In four instances, different type strains were recovered from these two sources.

DISCUSSION.

At the present time, the significance of the above findings is not definite, though certain explanations suggest themselves. Evidence has been accumulating which indicates that the mere presence of pathogenic pneumococci in the upper respiratory tract, or even in the lungs, is in the great majority of cases, possibly in all, not sufficient in itself to initiate the disease lobar pneumonia in man. It is true that Blake and Cecil (6) have shown that pneumonia may be induced in monkeys merely by the introduction of virulent pneumococci into the trachea, but the amount of culture employed was of considerable size, and in any case, in natural infection in man some preceding event would have to be postulated which would make it possible for the organisms to reach the trachea in sufficient numbers to initiate the disease. Furthermore, Blake and Cecil found it difficult to transmit Type I pneumonia from monkey to monkey by intimate and prolonged contact alone, only one out of six attempts being successful. They were also unable to initiate pneumonia in monkeys by the instillation of large amounts of a culture of virulent pneumococcus into the nose and throat, though the monkeys carried pneumococcus in the mouth for a month. From these observations, it seems likely that the mere presence of pneumococci in the normal upper respiratory tract, or even in the lung, can rarely, if ever, be held accountable for the onset of pneumonia.

More and more attention is therefore being given to the factors which are concerned in etiology previous to actual infection by the pneumococcus. It has been shown that 40 per cent of the cases of lobar pneumonia give a history of coryza (7) or other mild infection of the respiratory tract preceding the onset of pneumonia. It seems quite possible that a variety of contributing factors at present unknown, such as mild unrecognized infections, may act in lowering the local or general bodily resistance so that the pneumococcus may invade and infect the lungs.

Our observation that *Bacillus influenzæ* and pneumococci are frequently present in the nose and throat of pneumonia cases therefore suggests that these organisms may have been responsible for a coryza or infection of the mucous membranes of the nasopharynx preliminary to the infection of the lung with the pneumococcus. It must be borne in mind, however, that the relation of these organisms to ordinary colds is at present purely hypothetical. In any case, our observations indicate that in coryza and laryngitis, it is unusual for these organisms to be present in the nose.

Another possible explanation is that during the course of acute lobar pneumonia, the entire mucous membrane of the respiratory tract loses certain properties antagonistic to the growth of microorganisms, and that under these conditions all varieties of bacteria which normally grow sporadically are able to grow with great vigor. At present, however, the exact significance of our observation cannot be established and the above possibilities are offered merely as suggestions.

The fact that the hemoglobinophilic bacilli found in the upper respiratory tract of a given individual under the conditions discussed are not all of the same variety, is at first confusing and difficult to understand. One should bear in mind, however, the conditions in regard to the pneumococcus about which knowledge has been greatly increased during recent years. It is well known that pneumococci are of several types. Epidemiological evidence indicates that the Type IV pneumococci are relatively harmless and are normal inhabitants of the upper respiratory tract, while Type I and Type II pneumococci are almost always found in association with disease. It is not infrequent to find two types of pneumococci in one mouth, especially in the mouths of persons who have been in close association with pneumonia patients, but up to the present time the writer has never encountered a Type I and a Type II pneumococcus in the mouth at the same time. When pneumococci of one of these two types are present, together with pneumococci of Type IV, it is probable that the latter were present before the advent of the Type I or Type II pneumococci. Furthermore, the work of Olmstead (8) indicates that Type IV pneumococci may be further subdivided into definite types. Whether all the pneumococci of Type IV which are found in a single mouth are identical or not is not certain. It is not at all impossible that they may be of different kinds.

As regards hemoglobinophilic bacilli, the methods which have so far been proposed for separating them into groups or types cannot be said to be satisfactory. It is not possible at present to separate the saprophytic from the disease-producing varieties. The tentative classification based on indole production, gas formation, and saccharose fermentation which we have proposed may not be based on fundamental differences in the bacteria. When further knowledge concerning this group is obtained, the presence of several varieties in one mouth may possibly be shown to depend upon conditions analogous to those relating to pneumococcus.

SUMMARY.

1. *Bacillus influenzæ* is frequently found in association with lobar pneumonia.
2. In lobar pneumonia both *Bacillus influenzæ* and pneumococcus are frequently found in the nose.
3. The influenza bacilli found in the upper respiratory tract of cases of lobar pneumonia are of various types.
4. The exact significance of these findings is at the present time not clear.

BIBLIOGRAPHY.

1. Stillman, E. G., *J. Exp. Med.*, 1916, xxiv, 651; 1917, xxvi, 513. Pritchett, I. W., and Stillman, E. G., *J. Exp. Med.*, 1919, xxix, 259. Winchell, A. I., and Stillman, E. G., *J. Exp. Med.*, 1919, xxx, 497.
2. Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445. Avery, O. T., *J. Am. Med. Assn.*, 1918, lxxxi, 2050.
3. Park, W. H., and Wright, J., *J. Laryngol., Rhinol. and Otol.*, 1898, xiii, 124.
4. Allen, R. W., *Lancet*, 1908, ii, 1589.
5. Stillman, E. G., and Bourn, J. M., *J. Exp. Med.*, 1920, xxxii, 665.
6. Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.
7. Cole, R., in Nelson, Loose-leaf medicine. A perpetual system of living medicine, New York, 1920, i, 203.
8. Olmstead, M., *J. Immunol.*, 1916–17, ii, 425.

HETEROGENIC SERUM, AGE, AND MULTIPLICATION OF FIBROBLASTS.

BY ALEXIS CARREL, M.D., AND ALBERT H. EBELING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 21, 1921.)

I.

INTRODUCTION.

It has been shown that the inhibiting action of homogenic serum on the growth of a pure culture of chicken fibroblasts is directly proportional to the age of the animal from which the serum is taken, and that the rate of cell multiplication can be used as a reagent of certain changes produced by age in the blood serum.[1] Similar effects probably could be obtained from fibroblasts of mammals cultivated in homogenic serum. But it would be of advantage to cultivate chicken tissue in heterogenic serum because it is much more difficult to keep alive *in vitro* permanently a strain of mammal fibroblasts than of chicken fibroblasts. Over 10 years ago we found that chick embryo tissues grew abundantly in plasma from rabbits, dogs, and human beings, although not quite as well as in chicken plasma.[2] Later the effect of many heterogenic sera was investigated. Lambert and Hanes[3] cultivated rat sarcoma in guinea pig plasma and Ingebrigtsen[4] studied the action of several heterogenic sera on cultures of guinea pig bone marrow. The growth was always less rapid and extensive than in homogenic serum, and the toxic action of the serum varied according to the species of the animal. Owing to the lack of accuracy of the technique used at the time, no precise knowledge was gained of the action of these different sera.

---

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

[2] Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, xiv, 244.

[3] Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, xiv, 129.

[4] Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 397; xvi, 421.

A quantitative study of the influence of heterogenic sera, taken from animals of different ages, on the rate of growth of pure cultures of fibroblasts was begun by one of us in 1919. Fragments of a 7 year old strain of chicken fibroblasts were cultivated in media made of one volume of plasma and two volumes of serum taken from cats 1 month, 1 year, and 9 years old. The amount of growth obtained in the kitten serum was about four times greater than in the old cat serum. Similar experiments were made with serum obtained from human beings 22, 40, and 45 years old. Differences in the rate of growth were observed, although they were less marked than in cat serum. It became evident that the growth of chicken fibroblasts was more active in the serum of the young than in that of the old animals. When the technical improvements recently described by Ebeling rendered it possible to cultivate tissues in a medium composed of serum and fibrinogen,[5] and to prepare the cultures and measure the rate of growth with greater accuracy,[6] the study of the action of heterogenic serum was resumed. The purpose of the experiments described in this paper was to investigate the relation existing between the rate of growth of a pure culture of fibroblasts and the concentration in the medium of heterogenic serum, and the influence of the age of the animals from which the heterogenic serum was taken on the cell multiplication, in order to ascertain whether a pure culture of chicken fibroblasts could be used as a reagent for the detection of the modifications brought about by age in the blood of mammals.

II.

Action of Various Concentrations of Heterogenic Serum.

The action of different dilutions of homogenic and heterogenic sera on the rate of growth of chicken fibroblasts was thus investigated. The fibroblasts were taken from stock cultures of a 9 year old strain of chicken connective tissue, cultivated in one volume of embryonic tissue juice to two volumes of chicken plasma. Each fragment was divided in two equal parts, one being placed in the experimental medium and the other in the control. The experimental medium was composed of 20 per cent fibrinogen suspension, 5 per cent embryo juice, and varied amounts of Tyrode solution and dog or cat serum. The control medium had a similar composition, but the heterogenic serum was replaced by chicken serum. The fibrinogen suspension was prepared according to the method described by Ebeling.[5] The embryo juice was obtained from 8 or 9 day old chick embryos. Serum was taken from the plasma of cats or dogs under 2 years of age.

---

[5] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

[6] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

The action of media containing from 5 to 65 per cent heterogenic serum was compared with that of media containing identical amounts of chicken serum. Three cultures were made in each concentration of heterogenic serum. They were controlled by three cultures made in similar concentrations of chicken serum. The results of the experiments, showing the action of dog serum, were expressed by the relative increase of the tissues in 48 hours and are recorded in Tables I and II. The three experiments and controls of each series were averaged and the ratio, $\frac{\text{Relative increase in dog serum}}{\text{Relative increase in chicken serum}}$, was calculated. The variations in the rate of growth were expressed by a graph in which the ratio was plotted in ordinates and the serum concentration in abscissæ (Text-figs. 1 and 2). The rate of growth did not differ markedly from the control as long as the concentration of heterogenic sera was lower than 20 per cent. When the concentration of dog serum was increased from 20 to 35 per cent, the inhibiting action of the medium rapidly became more marked, and no growth was observed when the concentration reached 35 to 40 per cent. Other experiments showed that according to the individual, the serum concentration which prevents growth may vary from 30 to 45 per cent. The ratio of both relative increases in dog and chicken sera expressed the inhibiting action of dog serum on the proliferation of the fibroblasts, and can be called growth index of the serum. The growth index of the dog sera for a concentration of 30 per cent was respectively 0.32 and 0.067 (Tables I and II). The growth index of the cat sera for the same concentration (30 per cent) was 0.83 and 0.84. In other experiments, the growth index was also calculated in comparing the relative increases of a pure culture of fibroblasts in dog serum and in Tyrode solution, instead of chicken

TABLE I.

Experiment 1. Influence of Various Concentrations of Dog and Chicken Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.															
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.		Serum concentration 40 per cent.	
		Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.
1	21864	4.25	4.9														
2	21865	6.5	6.0														
3	21866	6.3	6.06														
4	21799			4.42	2.78												
5	21800			5.54	2.52												
6	21801			3.8	2.46												
7	21880			4.8	4.2												
8	21881			4.7	4.67												
9	21882			5.6	4.9												
10	21930					3.6	3.5										
11	21931					3.5	2.9										
12	21932					3.8	3.3										
13	21769							2.33	3.04								
14	21928							4.2	3.05								
15	21929							3.4	2.2								
16	21950							3.7	3.5								
17	21951							3.16	2.65								

18	21912									3.0	2.1						
19	21913									3.3	2.3						
20	21914									3.6	1.7						
21	21802											5.0	2.95				
22	21803											3.7	0.95				
23	21804											4.23	0.51				
24	21861													3.3	0		
25	21862													3.58	0		
26	21863													3.6	0		
27	21770															2.7	0
28	21910															5.5	0
29	21911															3.7	0
Average		5.68	5.65	4.81	3.59	3.63	3.2	3.36	2.88	3.3	2.0	4.31	1.47	3.49	0	3.96	0
Relative increase in dog serum Relative increase in chicken serum		0.96		0.75		0.89		0.93		0.62		0.32		0		0	

TABLE II.

Experiment 2. Influence of Various Concentrations of Dog and Chicken Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.													
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.	
		Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.	Chicken serum.	Dog serum.
1	21975	6.5	7.06												
2	21976	4.9	4.5												
3	21977	5.6	4.8												
4	21978			5.4	5.4										
5	21979			4.8	4.3										
6	21980			4.5	4.4										
7	21993					2.5	2.1								
8	21994					3.7	3.5								
9	21995					3.6	3.7								
10	21996							3.2	2.8						
11	21997							3.0	2.2						
12	21998							3.5	2.3						
13	22012									3.2	1.3				
14	22013									3.7	1.1				
15	22014									3.3	1.3				

16	22015											3.8	0.23		
17	22016											3.0	0.25		
18	22017											3.4	0.21		
19	22036													4.1	0.06
20	22037													3.6	0
21	22038													3.45	0
Average		5.66	5.45	4.9	4.7	3.26	3.1	3.2	2.43	3.4	1.2	3.4	0.23	3.7	0.02
Relative increase in dog serum Relative increase in chicken serum		0.96		0.96		0.98		0.75		0.36		0.067		0.005	

TABLE III.

Experiment 1. Influence of Various Concentrations of Cat and Chicken Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.													
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.	
		Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.
1	22052	5.70	5.20												
2	22053	7.80	6.50												
3	22054	5.50	4.90												
4	22055			5.00	4.90										
5	22056			4.70	4.70										
6	22057			5.60	5.50										
7	22063					4.26	4.00								
8	22064					4.70	4.56								
9	22065					4.50	4.70								
10	22066							4.35	4.80						
11	22067							5.05	5.20						
12	22068							5.50	5.40						
13	22084									3.68	3.65				
14	22085									4.19	4.00				
15	22086									4.20	4.14				

16	22087											4.30	3.70		
17	22088											3.93	2.86		
18	22089											3.26	3.00		
19	22153													3.15	2.38
20	22154													3.77	2.40
21	22155													3.73	2.53
Average		6.33	5.53	5.10	5.03	4.49	4.42	4.97	5.13	4.02	3.93	3.83	3.19	3.55	2.44
Relative increase in cat serum / Relative increase in chicken serum		0.87		0.99		0.98		1.03		0.98		0.83		0.69	

* The results obtained in higher dilutions were not recorded on account of errors due to liquefaction of the medium.

TABLE IV.

Experiment 2. Influence of Various Concentrations of Cat and Chicken Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.																							
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.		Serum concentration 40 per cent.		Serum concentration 45 per cent.		Serum concentration 50 per cent.		Serum concentration 55 per cent.		Serum concentration 60 per cent.	
		Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.	Chicken serum.	Cat serum.
1	22242–1 & 2	5.58	5.06																						
2	22243–1 & 2	4.56	4.69																						
3	22244–1 & 2	4.72	4.87																						
4	22245–1 & 2			4.90	4.70																				
5	22246–1 & 2			3.86	4.90																				
6	22247–1 & 2			4.56	4.44																				
7	22251–1 & 2					3.65	4.24																		
8	22252–1 & 2					3.96	4.24																		
9	22253–1 & 2					3.74	2.13																		
10	22254							4.09	4.26																
11	22255							4.30	3.18																
12	22256							3.55	2.83																
13	22257									3.57	3.02														
14	22258									3.57	3.24														
15	22259									3.00	2.83														
16	22266											3.85	3.18												
17	22267											4.14	3.26												
18	22268											3.84	3.48												

19	22269													4.27	2.80										
20	22270													3.91	3.25										
21	22271													4.09	3.00										
22	22272															3.60	2.17								
23	22273															3.03	2.08								
24	22274															4.18	2.39								
25	22287																	3.60	1.73						
26	22288																	3.65	1.71						
27	22289																	4.00	1.47						
28	22290																			3.75	0.83				
29	22291																			3.56	0.91				
30	22292																			3.44	1.13				
31	22310																					4.45	0.47		
32	22311																					3.50	0.19		
33	22312																					4.23	0.25		
34	22313																							4.05	No growth.
35	22314																							3.13	0.14
36	22315																							4.76	0.14
Average		4.95	4.87	4.44	4.68	3.78	3.54	3.98	3.42	3.38	3.03	3.94	3.30	4.09	3.02	3.60	2.21	3.75	1.64	3.58	0.96	4.06	0.30	3.98	0.09
Relative increase in cat serum Relative increase in chicken serum		0.99		1.06		0.94		0.86		0.89		0.84		0.74		0.61		0.44		0.27		0.074		0.023	

TABLE V.

Influence of Various Concentrations of Young and Old Cat Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.																					
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.		Serum concentration 40 per cent.		Serum concentration 45 per cent.		Serum concentration 50 per cent.		Serum concentration 55 per cent.	
		Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.
1	22299–1 & 2	5.30	4.83																				
2	22300–1 & 2	4.06	3.88																				
3	22301–1 & 2	3.61	3.47																				
4	22302–1 & 2			4.70	4.08																		
5	22303–1 & 2			4.33	4.58																		
6	22304–1 & 2			4.80	4.60																		
7	22359–1 & 2					5.47	4.70																
8	22360–1 & 2					4.35	3.88																
9	22361–1 & 2					4.75	4.21																
10	22362–1 & 2							4.32	4.67														
11	22363–1 & 2							5.02	4.56														
12	22364–1 & 2							5.12	4.06														
13	22365–1 & 2									4.93	4.65												
14	22366–1 & 2									4.80	4.12												
15	22367–1 & 2									4.15	2.98												
16	22382–1 & 2											3.52	2.94										
17	22383–1 & 2											3.66	2.98										
18	22384–1 & 2											3.10	2.56										

19	22385–1 & 2													4.00	2.90								
20	22386–1 & 2													3.30	2.23								
21	22387–1 & 2													2.35	2.00								
22	22388–1 & 2															2.37	1.91						
23	22389–1 & 2															2.32	1.15						
24	22390–1 & 2															2.76	1.19						
25	22443																	2.67	1.32				
26	22444																	2.40	1.00				
27	22445																	2.50	1.10				
28	22446																			2.10	0.64		
29	22447																			2.24	0.70		
30	22448																			2.11	0.73		
31	22449																					1.30	0
32	22450																					1.50	0
33	22451																					1.25	Few cells.
Average.		4.32	4.06	4.61	4.42	4.85	4.26	4.82	4.43	4.62	3.91	3.43	2.83	3.22	2.38	2.48	1.42	2.52	1.14	2.15	0.69	1.35	0
Relative increase in old serum / Relative increase in young serum		0.94		0.96		0.91		0.92		0.84		0.82		0.74		0.57		0.45		0.32		0	

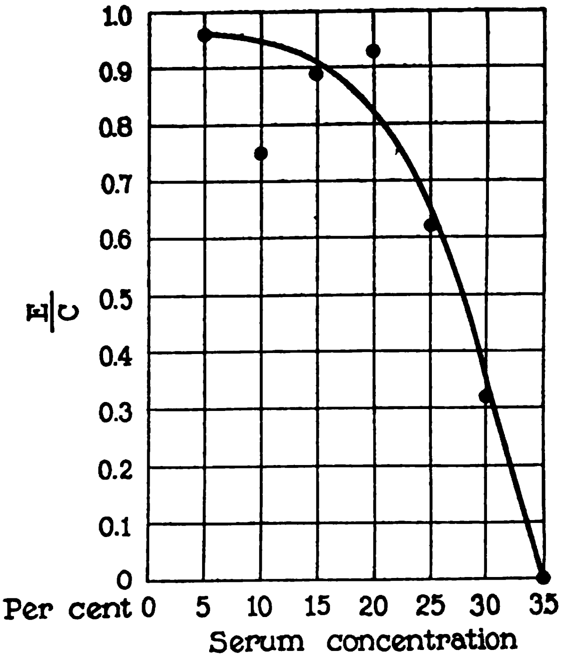
TABLE VI.

Influence of Various Concentrations of Young and Old Dog Sera on the Rate of Growth of Chicken Fibroblasts.

Experiment No.	Culture No.	Relative increase.																	
		Serum concentration 5 per cent.		Serum concentration 10 per cent.		Serum concentration 15 per cent.		Serum concentration 20 per cent.		Serum concentration 25 per cent.		Serum concentration 30 per cent.		Serum concentration 35 per cent.		Serum concentration 40 per cent.		Serum concentration 45 per cent.	
		Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.	Young serum.	Old serum.
1	22598	4.48	4.13																
2	22599	4.73	4.56																
3	22600	3.90	3.36																
4	22601			3.21	3.19														
5	22602			3.80	3.75														
6	22603			3.88	4.18														
7	22604					3.40	3.16												
8	22605					2.88	2.88												
9	22606					2.74	2.07												
10	22694							2.88	3.18										
11	22695							4.51	3.34										
12	22696							4.00	3.20										
13	22697									2.91	1.97								
14	22698									2.91	2.26								
15	22699									3.26	2.62								
16	22700											2.66	1.38						
17	22701											3.02	1.29						
18	22702											2.77	1.20						

19	22703													2.42	1.16				
20	22704													2.28	1.05				
21	22705													3.40	1.43				
22	22718															1.95	0.45		
23	22719															2.46	0.62		
24	22720															1.67	0.32		
25	22721																	1.10	0
26	22722																	1.57	0
27	22723																	1.50	0
Average		4.37	4.02	3.63	3.71	3.01	2.70	3.80	3.24	3.03	2.28	2.82	1.29	2.70	1.21	2.03	0.46	1.39	0
Relative increase in old serum / Relative increase in young serum		0.92		1.02		0.90		0.85		0.75		0.46		0.45		0.23		0	

serum. Similar experiments were made with cat serum and their results recorded in Tables III and IV, and in Text-figs. 3 and 4. The action of cat serum was not apparent until a concentration of 20 to 25 per cent was reached. Afterward, its action became stronger and no growth was observed when the concentration had reached 60 per cent.

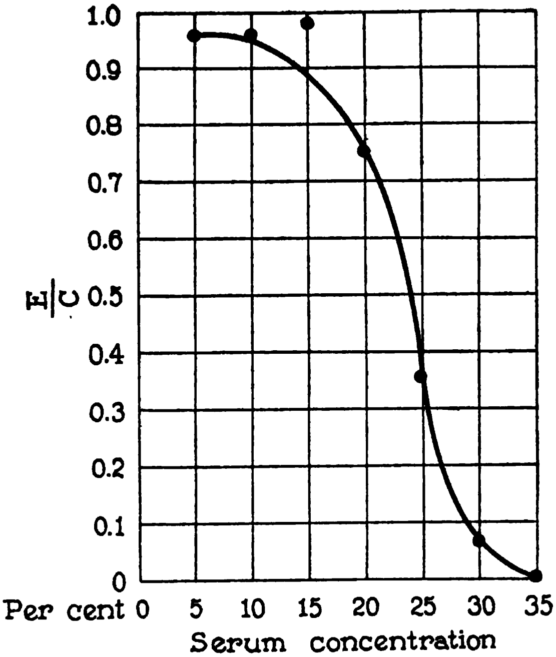


Text-Fig. 1. Experiment 1. Relation between the rate of growth of fibroblasts in dog and chicken sera. The ratio, $\frac{\text{Relative increase in dog serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

III.

Action of Heterogenic Sera from Young and Old Animals.

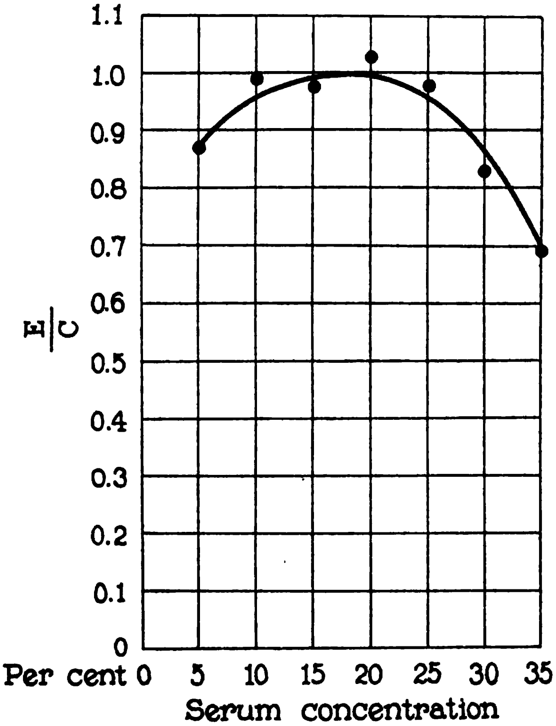
An examination was then made as to whether heterogenic sera from young and old animals would differ in their action on the growth of fibroblasts. Sera were obtained from cats 6 months and 9 years of age, and from dogs 6 months and about 10 years old. The technique was identical with that previously described. The relative increases of the cultures of fibroblasts in young and old cat sera were compared. In Table V are recorded the relative increase of the tissue in both media, and the quotient of the figures expressing the rate of multiplication in the old and young cat sera. In Text-fig. 5, the ratio, $\frac{\text{Relative increase in old serum}}{\text{Relative increase in young serum}}$, was plotted in ordinates and



TEXT-FIG. 2. Experiment 2. Relation between the rate of growth of fibroblasts in dog and chicken sera. The ratio, $\frac{\text{Relative increase in dog serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

the serum concentration in abscissæ. The graph shows that the action of both sera was about the same as long as the concentration present in the medium was not above 20 per cent. Afterward, the growth decreased more rapidly in the old animal serum. When the concentration reached 50 per cent, the rate of growth was only 32 per cent of that observed in a medium containing a similar amount of young cat serum.

The same experiments were repeated with sera obtained from a dog 6 months old, and from another about 10 years old. The relative increase of the cultures of fibroblasts in both sera is recorded in Table VI and Text-fig. 6, in which the ordinates represented the quotient of the rates of growth in old and young sera, and the abscissæ the serum concentration in the medium. The graph shows that the inhibiting action of the old dog serum was more marked than that of the young dog serum. When the concentration of the serum reached 40 per cent, the rate of growth in the serum of the old animal was only 23 per cent of the rate of growth in the serum of the young one.

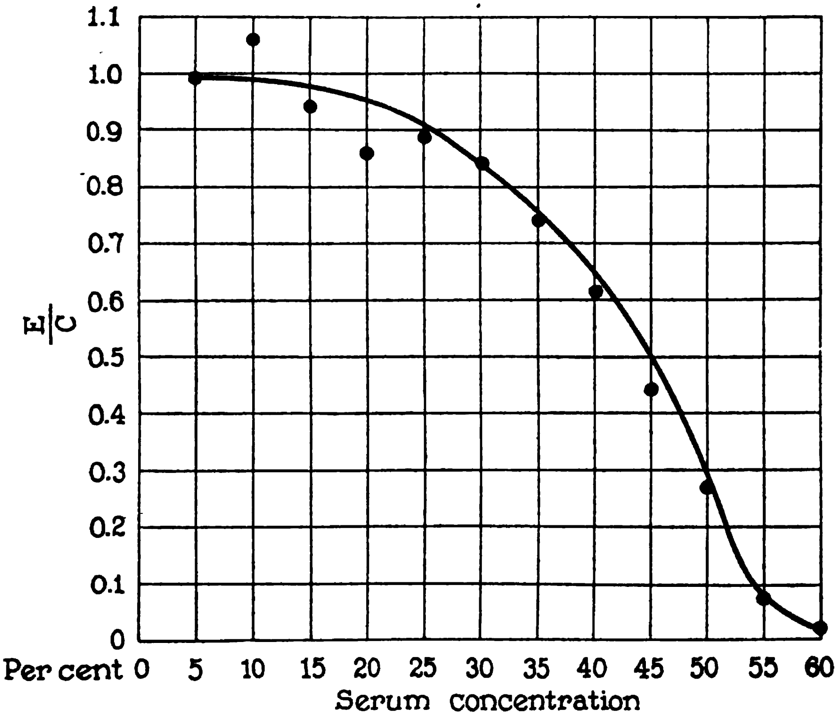


TEXT-FIG. 3. Experiment 1. Relation between the rate of growth of fibroblasts in cat and chicken sera. The ratio, $\frac{\text{Relative increase in cat serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

IV.

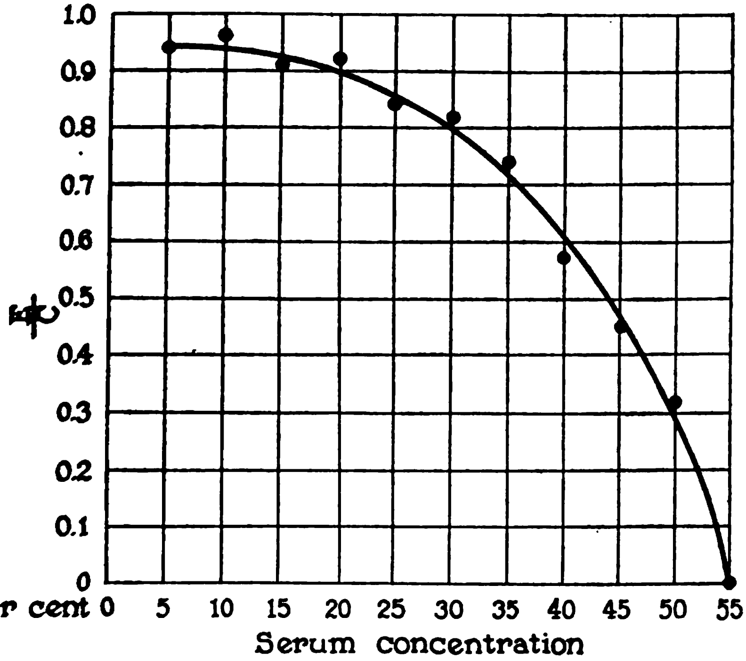
SUMMARY AND CONCLUSIONS.

The presence in a culture medium of heterogenic serum of various concentrations exerts a definite influence on the rate of multiplication of fibroblasts. Dog serum does not inhibit the growth of chicken fibroblasts markedly until its concentration reaches 15 per cent. Beyond this figure, each increase of the concentration brings about a rapid decrease in the rate of cell multiplication. When the concentration reaches from 30 to 45 per cent, no growth takes place. The inhibiting action of cat serum begins to manifest itself at a concentration of 25 per cent and prevents cell prolifera-



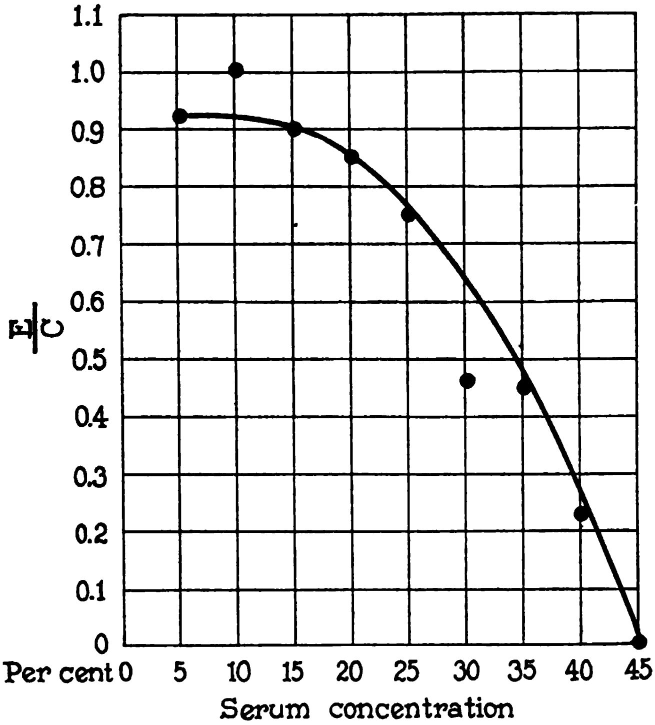
TEXT-FIG. 4. Experiment 2. Relation between the rate of growth of fibroblasts in cat and chicken sera. The ratio, $\frac{\text{Relative increase in cat serum}}{\text{Relative increase in chicken serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

tion completely at a concentration of 55 and 60 per cent. The ratio, $\frac{\text{Rate of growth in heterogenic serum}}{\text{Rate of growth in chicken serum}}$, can be taken as expressing the action of the serum on fibroblast multiplication; that is, as the growth index of the serum.



Text-Fig. 5. Relation between the rate of growth of fibroblasts in young and old cat sera. The ratio, $\frac{\text{Relative increase in old serum}}{\text{Relative increase in young serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

The inhibiting influence of heterogenic serum was found to vary in direct ratio to the age of the animal from which it was obtained. The rate of proliferation of chicken fibroblasts was studied comparatively in media containing varied concentrations of serum from young and old animals. For each concentration of serum, the rate of growth in the serum of the old animal was expressed in relation to the rate of growth in the serum of the young animal. When cat serum was used, the curve obtained in plotting this ratio in ordinates and the serum concentration in abscissæ showed a rapid increase in the inhibiting action of the old serum as soon as the concentration reached 30 per cent. The same tests were repeated with the serum from young and old dogs. The general results were identical, although the quantitative inhibiting action of both sera was greater than that of cat serum. It may be concluded that under the conditions of the experiments:



TEXT-FIG. 6. Relation between the rate of growth of fibroblasts, in young and old dog sera. The ratio, $\frac{\text{Relative increase in old serum}}{\text{Relative increase in young serum}}$, is plotted in ordinates and the serum concentration in abscissæ.

1. Heterogenic sera inhibit and prevent the growth of chicken fibroblasts when their concentration is made to vary within certain limits.

2. A relation exists between the rate of growth of chicken fibroblasts, the concentration of the heterogenic serum, and the age of the animal from which the serum is taken. Thus, the rate of proliferation of chicken fibroblasts expressed by the growth index of the serum can be used as a means for detecting certain changes brought about by age in a heterogenic serum.

A STUDY OF THE RELATION OF TREPONEMA PALLIDUM TO LYMPHOID TISSUES IN EXPERIMENTAL SYPHILIS.

BY LOUISE PEARCE, M.D., AND WADE H. BROWN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 27, 1921.)

The establishment of the fact that dissemination of *Treponema pallidum* in the rabbit occurs after local inoculation has for the most part rested upon the evidence of the development of syphilitic lesions remote from the site of the primary lesion and upon occasional demonstrations of spirochetes in certain tissues, such as the spleen, blood, bone marrow, and lymph nodes. Our experience of the frequency of generalized lesions following inoculation of testicle or scrotum has differed from that of most workers on experimental syphilis, and in addition we have shown that it is possible to produce generalized lesions with great regularity by simple technical procedures either at the time of infection or shortly thereafter so that under certain conditions at least, dissemination of *Treponema pallidum* is known to have occurred in every animal infected (1). The mechanism of the process, however, is still undetermined, and before the question of a generalized infection can be put upon a logical basis, it is necessary that such essential facts as the time, frequency, and extent of dissemination under the usual conditions of inoculation procedure together with the localization and extent of the infection should be obtained. Among the possible modes or paths of dissemination which might be assumed to participate in the process of generalization, are the lymphatic and blood systems, and both are easily accessible to experimental investigation, as indicated in a preliminary communication (2).

Among the generalized manifestations of experimental syphilis in the rabbit, enlargement and induration of various lymph nodes are commonly observed. The superficial nodes which most frequently show various degrees of involvement after the usual scrotal or sheath inoculation are the inguinals, and similar changes are seen in the sacral retroperitoneal glands following testicular inoculations. Involvement of other nodes such as the flank, axillary, auricular, cervical, and submental glands has also been observed in certain animals, and apparently some abnormality of the popliteals is a constant feature of the infection irrespective of the site of inoculation.

Such changes as take place, however, might logically be expected to appear first in the lymph nodes draining the area of the primary reaction if dissemination of spirochetes from a local focus of inoculation regularly occurs by way of the lymphatics, and consequently the problem was first approached by a series of experiments designed to show the presence or absence of *Treponema pallidum* in the inguinal lymph nodes of rabbits inoculated in the scrotum.

The studies of the part played by the blood stream in the process of dissemination of *Treponema pallidum* are reserved for a later publication, but it should be stated here that before it could be concluded that organisms were localized in lymphatic tissue, it was necessary to exclude the blood as a possible source of spirochetes. During the early stages of the infection, this could be done only upon the basis of relative infectivity. Later, however, blood inoculations were uniformly negative. It is obvious, moreover, that a blood stream infection must be ruled out before one can conclude that spirochetes are localized in any other tissue, as for instance in the spleen or bone marrow.

EXPERIMENTAL.

The inguinal lymph nodes of normal rabbits are small, flattened or ovoid masses which are barely visible or palpable and are frequently imbedded in relatively large pads of fat which renders them more difficult of detection. Gross changes in the nodes are usually detected within 4 or 5 days after scrotal inoculation and consist in a slight enlargement together with an increased resistance or tension.[1] As the process continues, the glands rapidly become indurated and many times their normal size, and, in our experience, show some degree of abnormality as long as an active syphilitic process exists in the scrotum.

---

[1] This was the condition at the time these experiments were carried out. Subsequently, however, definite alterations of the nodes have frequently occurred within 24 hours after scrotal or sheath inoculation.

The demonstration of *Treponema pallidum* in lymph nodes may be accomplished by two methods—dark-field examination of fluid aspirated from the node or by animal inoculation. Direct aspiration of lymph nodes *in situ* by means of a syringe is a difficult procedure, particularly in the early involvement of the nodes, and the fluid obtained is often contaminated with blood. Moreover, as will be pointed out later, such a procedure may necessitate the examination of many preparations, and, therefore, we had recourse to the second and more satisfactory method which consisted in the removal of an inguinal node under ether anesthesia, the making of a node emulsion with salt solution, and the injection of this emulsion into the testicles of normal rabbits.

The extirpated lymph node is first minced with fine scissors and then ground in a sterile mortar with a small quantity of sterile normal salt solution. In most of our experiments we used a total quantity of 1.5 cc. of saline solution and were able to recover 1.2 cc. of the emulsion; 0.6 cc. was then injected into one testicle of each of two normal rabbits by means of a Fournier tuberculin syringe and a No. 22 gauge Luer needle. The subinoculated rabbits were closely followed during an observation period of 2 months for the development of syphilitic lesions. The organisms used in these experiments were the Zinsser and the Nichols strains of *Treponema pallidum* which have been maintained in rabbits for periods of 7 to 8 years.

Results.

The Frequency and Time of Inguinal Lymph Node Involvement.

Our preliminary experiments were concerned with the demonstration of the presence of *Treponema pallidum* in the inguinal lymph nodes of a few rabbits with large actively growing chancres. The results of the individual experiments are grouped together, and, as may be seen in Table I, the interval of time after scrotal inoculation at which the six experiments were carried out was progressively shortened from 61 to 16 days. The chancres were all well developed, while the inguinal glands were many times normal size, and markedly indurated. One gland was excised from each rabbit. Although spirochetes were found by dark-field examination in but one gland emulsion a syphilitic orchitis developed in all the subinoculated rabbits in from 15 to 41 days as a result of the testicular injection of the lymphatic gland emulsion, showing that there had been an actual dissemination of spirochetes in each instance from the local focus of infection in the scrotum.

TABLE I.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 61 to 16 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	Interval between scrotal inoculation and excision of inguinal lymph node.	Examination of inguinal node emulsion for spirochetes.	Rabbit inoculated with lymph node emulsion.		Result of inoculation.	Incubation period of orchitis.
			No.	Testicle.		
	days					*days*
A	61	Negative.	1	Right.	+	41
				Left.	+	
B	49	"	2	Right.	+	Exact time
				Left.	+	not known.
C	35	Positive.	3	Right.	+	15
				Left.	+	33
D	35	Negative.	4	Right.	+	33
				Left.	+	33
E	18	"	5	Right.	+	27
				Left.	+	28
			6	Right.	+	26
				Left.	+	28
F	16	"	7	Right.	+	34
				Left.	+	34
			8	Right.	+	26
				Left.	+	28
Total.6						

+ indicates infection.

In the preliminary experiments, one rabbit was used for each gland emulsion and both testicles were inoculated, but later, the precaution of using two rabbits was adopted. In the majority of experiments, one testicle only was inoculated.

Our next experiment was planned to determine the incidence of infection of the inguinal nodes 7 days after scrotal inoculation, when enlargement and increased resistance of the nodes are found in practically every instance, although the specific reaction about the implant is usually just beginning. The results of the experiment are shown in Table II.

TABLE II.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 7 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	Rabbit inoculated with lymph node emulsion.		Result of inoculation.	Incubation period of orchitis.
	No.	Testicle.		
				days
G	9	Right.	+	28
		Left.	+	28
	10	Right.	+	28
		Left.	+	28
H	11*	Right.	+	35
	12*	"	+	33
I	13*	"	+	35
	14*	"	+	35
J	15	"	+	30
	16	Accidental death.		
K	Spirochetes +; no inoculation.			
L	" +; " "			
Total...6				Average..32

* Animals of poor quality with very small testicles.
+ indicates infection.

A series of six rabbits was operated upon and one of the inguinal glands removed. Dark-field examination of the gland emulsions showed actively motile spirochetes in two instances and no subinoculations were made from these; the remaining four emulsions were injected into the testicles of eight normal rabbits. The injection of one emulsion into both testicles of two rabbits resulted in an acute orchitis which developed simultaneously in 28 days in the four testicles, while the remaining three emulsions were injected into one testicle each of six rabbits. There was one accidental death soon after inoculation, but in the surviving five animals, a syphilitic orchitis developed in from 30 to 35 days and progressed actively in the usual fashion.

The consistently positive results of this experiment were most striking in that all six animals examined 7 days after scrotal inoculation were found to have infected inguinal glands in spite of the fact that a specific reaction about the scrotal implant could scarcely be detected.

In the next experiment, the interval of time between scrotal inoculation and extirpation of the inguinal node was shortened to 5 days (Table III).

The saline emulsions of ten inguinal glands were injected into the testicles of eighteen normal rabbits; in two instances, one subinoculation was made, but in eight, two rabbits were used. One bilateral inoculation was made. Spirochetes were not found by dark-field examination in any of the gland emulsions. A syphilitic orchitis, however, developed in seventeen of the eighteen test rabbits with an average incubation period of 30 days; in two instances, the orchitis was detected 25 days and in five instances, 28 days after inoculation. In sixteen of the eighteen rabbits, the reaction was of a relatively rapid progressive type, while in one rabbit with very small atrophic testicles, it was not particularly active and tended toward a localized area of induration. The orchitis which resulted from the single bilateral inoculation in the experiment developed in 33 days in one testicle and 39 days in the other, which was the longest incubation period in the series. In one animal a progressive orchitis failed to develop during the observation period of 8 weeks, and it is possible that this was an instance of an atrophic orchitis; the companion rabbit, however, inoculated with the same material, showed the usual reaction with an incubation period of 34 days.

The results of this experiment coincide to a remarkable degree with those obtained in the previous series and demonstrate in a considerable number of animals that the inguinal lymph nodes of rabbits inoculated in the scrotum only 5 days previously and at a time when no specific reaction about the implant was observed were constantly infective. In addition, although no spirochetes were demonstrated in the lymph gland emulsion by dark-field examination, the material was sufficiently potent to produce an actively developing syphilitic orchitis with an average incubation period of 30 days.

Finally, a similar experiment was undertaken 2 days after scrotal implantation when no gross alteration of the inguinal nodes was detected by palpation or by inspection, since the previous experiments had shown that enlargement and tenseness of the nodes, 5 and 7 days after scrotal inoculation, were constantly associated with the presence of *Treponema pallidum*.

TABLE III.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 5 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	No. of rabbit inoculated with lymph node emulsion.	Result of inoculation.*	Incubation period of orchitis.
			days
M	17	+	33
	18	+	30
N	19	+	28
O	20†	?	?
	21	+	34
P	22	+	33
Q	23‡	+	33 and 39
	24	+	28
R	25	+	25
	26	+	33
S	27	+	33
	28	+	25
T	29	+	33
	30	+	29
U	31	+	28
	32	+	28
V	33	+	30
	34	+	28
Total..........10	18		Average........30

* Right testicle inoculated.

† Rabbit 20 was an old animal with small testicles, and the reaction was of the atrophic type.

‡ Rabbit 23 was inoculated into both testicles with positive results in 33 and 39 days.

+ indicates infection.

One inguinal node was excised from seven rabbits, five of which had been inoculated 2 days previously with the usual tissue implant and two with a testicular emulsion injected into the scrotum. Salt solution emulsions of the nodes were made as in the previous experiments and injected into the right testicle of twelve normal rabbits as shown in Table IV. Although no spirochetes were found in any of the emulsions by dark-field examination, a marked syphilitic orchitis developed in all subinoculated rabbits with an average incubation time of 31 days. The inguinal glands which were removed from rabbits with tissue implants produced an orchitis which was detected 28 and 29 days after inoculation, with the

TABLE IV.

Demonstration of Treponema pallidum in the Inguinal Lymph Nodes 2 Days after Scrotal Inoculation.

No. of rabbit with chancre implant.	No. of rabbit inoculated with lymph node emulsion.	Result of inoculation.*	Incubation period of orchitis.
			days
W	35	+	28
	36	+	30
X	37	+	28
Y	38†	+	38
	39†	+	36
Z	40	+	29
	41	+	29
AA	42	+	29
	43	+	29
BB‡	44	+	30
	45	+	33
CC‡	46	+	33
Total..........7	12		Average........31

* Right testicle inoculated.

† Rabbits 38 and 39 were old animals with atrophic testicles which probably accounts in part at least for the longer incubation time of the orchitis.

‡ Rabbits BB and CC were inoculated by a scrotal injection of an orchitis emulsion instead of the usual tissue implant.

+ indicates infection.

exception of two old animals with atrophic and less reactive testicles in which the orchitis developed more slowly with incubation periods of 36 and 38 days. The orchitis produced by the injection of the lymph nodes obtained from the two rabbits inoculated with a tissue emulsion was slightly longer, developing in 30 and 33 days, but obviously a much larger series would be necessary before any final comparisons could be made between the relative infectivity of the nodes resulting from the two types of scrotal inoculation.

The outstanding features of this experiment are first, the demonstration that only 48 hours after inoculation of the scrotum, the inguinal nodes contain active *Treponema pallidum*, and second, that the treponemata were constantly present in the inguinal nodes at this time. It is also worthy of comment that although no spirochetes could be demonstrated in the inguinal gland emulsions by dark-field examination, the orchitis resulting from the injection of the emulsions was of an actively developing type with an average incubation period of 31 days, and in half the cases of 28 and 29 days.

Time of Establishment of a Generalized Infection.

The previous experiments have shown that active treponemata capable of producing a rapidly progressive orchitis were constantly present in the inguinal lymphatic nodes of twenty-nine rabbits, 2 to 61 days after scrotal inoculation.

The question that next arose concerned the time of establishment of a generalized infection, since the inguinal glands are so rapidly infected after scrotal implantation although the development of the specific chancre reaction itself is usually not recognizable for some 10 to 14 days (3). In reality it is conceivable that many parts of the animal body might be infected shortly after local inoculation and that they in turn might serve as other foci for further dissemination of the virus. At any rate, we know that the inguinal nodes contain active treponemata as early as 2 days after scrotal inoculation, but on the other hand, such organisms might be chance invaders which had been rapidly disseminated from the primary focus of infection and, as such, would be incapable of independently sustaining an infection. Since there was no available information on these points, the following experiment was undertaken.

A series of ten rabbits was inoculated with tissue implants in the right scrotum only. 2 days later, under ether anesthesia, the entire right testicle with the scrotum was excised, leaving only a narrow circular strip of the posterior scrotal skin with which to close the wound. Healing took place promptly in each animal with no secondary infection. At the time of operation, no difference in the two inguinal lymph nodes could be made out by palpation and no specific reaction about the implant had occurred. The rabbits were kept under careful observation and the results of the experiments are shown in Table V. 40 days after operation, and 42 days after scrotal inoculation, a definite syphilitic lesion was present in the left testicle or head of the epididymis in four rabbits and a suspicious lesion in three others. By the end of the 7th week after inoculation, the left testicle of all ten rabbits was the seat of a progressive nodular orchitis. In addition, definite periosteal nodules of the nasal bones in four cases and a skin granuloma on the outer side of the right tarsus in one case were also found at the time; *i.e.*, 45 days after inoculation. Bone lesions were found in four other rabbits 4 days later and in another animal by the end of the 8th week, while the last rabbit in the group showed definite bone lesions 75 days after inoculation. Other generalized manifestations consisting of cutaneous granulomata were observed in five rabbits during the 2 months succeeding the appearance of the first lesions.

TABLE V.

Results Following Unilateral Amputation of the Right Scrotum and Castration of the Right Testicle, 2 Days after Inoculation of the Right Scrotum.

No. of rabbit.	Development of syphilitic lesions in left testicle.	Interval between inoculation and appearance of testicular lesions.	Presence of popliteal lymphadenitis.	Lesions of generalized syphilis, other than testicular.	Interval between inoculation and appearance of generalized lesions.
		days			*days*
A	+	42	+	Bone.	49
B	+	42	+	Skin.*	42
C	+	49†	+	Bone.	49
D	+	49†	+	“	45
E	+	49‡	+	“ § skin.	45
F	+	42	+	“	49
G	+	42	+	“	75
H	+	49†	+	“	56
I	+	49‡	+	“	45
J	+	49‡	+	“	45

* Scrotal lesion.

† Suspicious orchitis—42 days.

‡ Orchitis of 3 or 4 days standing on the 49th day.

§ Lesion, right tarsus.

The right inguinal lymph nodes were definitely enlarged and resistant for some 10 days after the operation and then became shot-like in character. Both popliteal nodes were likewise persistently enlarged and indurated in each rabbit of the series, this change being pronounced by the time of occurrence of the metastatic testicular lesions.

The consistent results of this experiment demonstrate that 2 days after scrotal inoculation an active syphilitic infection is sufficiently established in the rabbit so that it is no longer dependent upon the focus of primary inoculation as a source of spirochetes.

Localization of Spirochetes in Lymph Nodes Other than the Inguinals.

Other superficial lymph structures such as the popliteal, cervical, submental, auricular, axillary, and flank nodes may also become enlarged and indurated during the course of syphilitic infection of the rabbit following local inoculation, but with the exception of regional lymph nodes in the drainage area of an active lesion, the popliteals show the most conspicuous and persistent abnormalities, of which a resistance or induration is usually the most marked feature. In many instances, the popliteals may be the only nodes in which gross abnormalities are detected. In order to demonstrate the presence of *Treponema pallidum* in lymph nodes other than the inguinals which showed these clinical changes, a number of animal inoculation experiments were carried out, of which the following four examples are typical.

Rabbit A.—Inoculated in the right testicle with an inguinal lymph node emulsion. A syphilitic orchitis developed and spirochetes were demonstrated in fluid aspirated from the testicle 41 days after inoculation. Both popliteals were enlarged and resistant, and 51 days after inoculation, the left node was resected under ether anesthesia, emulsified with salt solution, and injected into the right testicle of two normal rabbits. At this time, there were no generalized syphilitic lesions and no spirochetes were seen in the lymph gland emulsion by dark-field examination. One of the subinoculated rabbits died from accidental causes, but in the surviving animal, a syphilitic orchitis, in which spirochetes were easily demonstrated, developed, with an incubation period of 43 days.

Rabbit B.—Bilateral scrotal inoculation. At the time of operation, 14½ months later, there were active skin granulomata on the outer side of the right hind foot, left ankle, and nose, and extensive residual ocular changes, not, however, active at this time. Both popliteals were extremely large and rather resistant with some fluctuation. The right node was aspirated with a syringe and 2 cc. of opalescent fluid flowed into the syringe without suction. No motile spirochetes were seen by dark-field examination of this fluid, of which 0.5 cc. was injected into the testicles of two normal rabbits. A syphilitic orchitis subsequently developed in both rabbits with incubation periods of 39 and 42 days.

Rabbit C.—Bilateral testicular inoculation, with the popliteal lymph node of Rabbit B. At the time of operation, 3 months later, there was a nodular orchitis of 46 days duration and a keratitis in the right eye. Right popliteal lymph node slightly enlarged and indurated. Left popliteal lymph node, however, which had been enlarged and indurated for some weeks, was resected under ether anesthesia, emulsified with salt solution, and 0.5 cc. was injected into the testicles of two normal rabbits. No spirochetes were seen in the emulsion by dark-field examination. An orchitis, however, was detected in the first subinoculated rabbit in 22 days, with subsequent demonstration of spirochetes, and progressed in an active manner. The second subinoculated animal was discarded because of a subcutaneous abscess.

Rabbit D.—Bilateral scrotal inoculation. At the time of operation, 16 months later, there were cutaneous, subcutaneous, and periosteal nodules of the lower portion of the nose and a large cutaneous granuloma of the left ankle. A right axillary lymph node and one in the upper left flank had been enlarged and indurated for some months. Enlarged cervical lymph nodes consisting of several firm, fairly elastic nodules in the deeper tissues of the neck and one large node under the right maxilla near the midline had also been observed for a considerable period of time. The large submental node was aspirated with a syringe and 0.4 cc. of an opalescent fluid was withdrawn. No spirochetes could be demonstrated by dark-field examination. The fluid was injected into the right testicle of a normal rabbit, and a nodular syphilitic orchitis subsequently developed with an incubation period of 51 days.

These experiments demonstrate that during the course of the syphilitic infection of rabbits produced by local inoculation of testicle or scrotum, superficial lymph nodes are infected with *Treponema pallidum*. In Rabbit A, there were no generalized lesions, and in Rabbit C, although there was a keratitis, there were no lesions about the hind feet and legs at the time the popliteal node was excised, so that the presence of *Treponema pallidum* in these nodes cannot be ascribed to a drainage infection. In the case of Rabbit B, however, there were active syphilitic lesions of the lower extremities and about the nose, so that the factor of drainage must be included in the infection of the regional lymph nodes in this instance. Clinically, this added factor is usually expressed by the larger size and the tenseness of the lymph nodes rather than by induration. In Rabbit B, an axillary and an upper flank node were also enlarged and indurated, a condition which was undoubtedly due to the presence of *Treponema pallidum.*

Duration of Lymph Node Infection.

The presence or absence of spirochetes in lymph glands during the various phases of syphilitic infection in the rabbit is of particular interest and importance and especially during the so called latent period in which no syphilitic lesions can be found. As we have pointed out in a previous paper (4), spontaneous recovery in the rabbit is frequently only temporary, and lesions of some kind may recur after the lapse of a few months and occasionally of more than a year. Such intervals are examples of true latency in which the rabbit is still infected, as evidenced by subsequent syphilitic lesions, but during which the animal organism is able, for the time being, both to restrain the progressive development of spirochetes and to protect itself from their harmful effects. During this latent period, there is but one condition to indicate that infection may still exist, and that is the presence of some degree of abnormality of the superficial lymph nodes, especially the popliteals. The changes noted in cases of latent infection vary from a shotty induration of small nodes to a moderate enlargement with some degree of induration. The method of animal inoculation was used to ascertain whether spirochetes were present in the lymph nodes of rabbits in which the infection was latent.

The eight rabbits used for this series of experiments had been locally inoculated 7 months to 4½ years previously as is shown in Table VI. All had had characteristic local lesions, and the first six animals in the table had shown well marked generalized lesions. The eighth animal was a female whose inoculation on the vulva resulted in a typical chancre, but no succeeding generalized manifestations had developed. At the time of operation, six rabbits showed no lesions of any kind, while two showed slight lesions of an indifferent character in which no spirochetes could be demonstrated by dark-field examination, and it was subsequently shown that one of these was not syphilitic (5). The latent periods of infection in these animals varied from 3 to 25 months. All eight rabbits, however, showed a popliteal adenopathy varying from small shot-like glands which were atrophic and fibrous to moderately enlarged and indurated glands which showed some degree of medullary swelling. The popliteal nodes were resected under ether anesthesia, emulsified with salt solution, and 0.5 cc. of the emulsion was injected into the right testicle of two normal animals.

The results of these experiments demonstrate the constant presence of *Treponema pallidum* in the popliteal nodes of rabbits during latent periods of a syphilitic infection. Moreover, the rapidity with which an orchitis developed in the subinoculated rabbits, with incubation periods of 31 to 44 days, is particularly striking. In both of these respects, the results practically coincide with those obtained from the inoculation of lymph gland emulsions from cases of acute or active syphilis, as shown in the preceding sections of this paper. The duration of infection or of the period of latency in the group of animals reported is not unusual, except in Rabbits A, G, and H. The infection in these animals had at all times been mild; only a few lesions developed and these were comparatively slight. Nevertheless, virulent organisms remained alive in Rabbits A and G for approximately 4½ and 3 years. Rabbit A is by far the longest case of animal infection on record and indicates very conclusively that the apparent recovery which takes place in these animals is only a symptomatic one.

The subsequent history of the eight rabbits extends over many months. Five animals have shown no change whatever in their condition. In Rabbit D, a slight infiltration about the right nostril extended to the left and eventually presented the typical appearance of syphilitic lesions in this locality, while in Rabbit E, a keratitis developed later. In the case of Rabbit A, the lesion in the scrotum proved to be a malignant growth from which extensive metastases occurred to the right and left inguinal and left flank lymph nodes, lungs, liver, spleen, kidneys, and bone marrow, leading eventually to marked emaciation, asthenia, loss of sphincter control, and the development of trophic ulcers about the anus, so that the animal was killed.

It would seem, therefore, from the striking results of these experiments, that for long periods of time, *Treponema pallidum* may be present in the lymph nodes of rabbits which have recovered from clinical manifestations of the disease even though no further manifestations of the infection occur, and further that the reservoir of the organisms during latent periods of syphilitic infection is lymphoid tissue.

The Use of Lymph Node Reservoirs as a Means of Demonstrating Infection.

The data obtained from the various series of experiments described in this paper indicate the fundamental rôle played by lymphatic tissue in syphilitic infection of the rabbit. We know that dissemination of spirochetes from a local focus of inoculation in the scrotum to the inguinal lymph nodes occurs extremely rapidly and that during the course of an infection *Treponema pallidum* may be recovered from other superficial lymph nodes as well. This may be accomplished not only during active phases of the infection but in the so called latent period in which no manifestations of the disease are found except abnormal popliteal lymph nodes which contain treponemata of surprising infectivity.

Up to the present time, there has been no means at one's disposal to ascertain whether or not infection still persists in a rabbit if no local recurrence or generalized lesions develop, except to hold the animal for a prolonged period of time for such manifestations to appear. This has been the method employed in judging the results of the treatment of the experimental infection, but the use of lymph node inoculations offers an illuminating as well as a time-saving procedure for determining the ultimate or curative effect of a therapeutic agent, as illustrated by the following experiment.

Among a group of rabbits treated with arsphenamine and neoarsphenamine, there were five which showed no clinical recurrence during a period of 3 months observation. These animals had been inoculated in both testicles with the Nichols strain of *Treponema pallidum*; two rabbits were given single doses of 6 mg. per kilo of arsphenamine (German salvarsan Lot A 25819) and three rabbits 9 mg. per kilo of neóarsphenamine (German neosalvarsan, Lot A 25884). The drugs were administered intravenously in a 0.2 per cent solution; the arsphenamine was neutralized by the theoretical amount of N sodium hydroxide to form the disodium salt. The doses employed have been shown to produce definite therapeutic effects in the average animal, consisting of a regression of lesions approximating complete resolution with freedom from recurrence for 4 to 6 weeks followed by clinical relapse within a period of not more than 2 to 3 months (6).

The immediate therapeutic effects consisted in the regression and resolution of the testicular lesions, which proceeded somewhat more rapidly in those animals treated with neoarsphenamine, so that by the end of the 2nd week, the testicles were normal except for small residual areas of thickening or infiltration in four animals, which continued unchanged during the 3 months observation period following treatment.

TABLE VI.

Demonstration of Treponema pallidum in the Popliteal Lymph Nodes during Latent Periods of Infection.

Source rabbit.	Duration of infection.	Length of latent period.	No. of rabbit inoculated with popliteal lymph node emulsion.	Result of inoculation.	Incubation period of orchitis.
	mos.	*mos.*			*days*
A*	51	6	1	+	38
			2	+	43
B	9	3	3	+	42
			4	+	44
C	7	3	5	+	37
			6	+	39
D*	9	3	7	+	34
			8	+	31
E	7	3	9	+	37
			10	Dead.	
F	7	3	11	+	31
			12	+	31
G	35	25	13	+	35
			14	+	35
H	10	7	15	+	34
			16	+	34

* These two animals showed suggestive lesions at the time of examination. They subsequently increased but no spirochetes could be demonstrated in them by dark-field examination. The lesion in the case of Rabbit A proved to be a malignant growth.

+ indicates infection.

Aspiration of the testicles for dark-field examination was not done because of the possible regressive effect of such a trauma. In addition no generalized manifestations of a specific character had developed. The only suggestive evidence of the existence of a still active infection 3 months after treatment was an enlargement and induration of the popliteal lymph nodes which were present in all five rabbits. In order to determine definitely the presence or absence of infection, the left popliteal node was excised for inoculation into test animals. Upon removal, the nodes showed a slight degree of enlargement, slight fibrosis of the surrounding tissue, and slight thickening of the capsule; they were translucent and firm with prominent lobulation. A salt solution emulsion of each node was prepared and from 0.2 to 0.4 cc. was injected into the right testicle of two normal rabbits as shown in Table VII.

TABLE VII.

Demonstration of Treponema pallidum in the Popliteal Lymph Nodes 3 Months after Treatment with Arsphenamine and Neoarsphenamine.

Source rabbit.*	Interval between inoculation and treatment.	Treatment; amount per kilo.	Amount of popliteal lymph node emulsion injected.	No. of rabbit inoculated with popliteal lymph node emulsion.	Result of inoculation.	Incubation period of orchitis.
	days		*cc.*			*days*
A	18	6.0 mg. of arsphenamine.	0.3	1	+	37
			0.3	2	+	45
B	18	6.0 " " "	0.3	3	+	34
			0.3	4	+	34
C	18	9.0 mg. of neoarsphenamine.	0.4	5	+	34
			0.4	6	+	34
D	18	9.0 " " "	0.2	7	+	29
			0.2	8	+	31
E	18	9.0 " " "	0.4	9	+	29
			0.4	10	+	31

* All five rabbits presented a progressive nodular orchitis which was approximately at the height of its acute phase or cycle at the time of treatment.

+ indicates infection.

The noteworthy features of this series of experiments are first, the constancy with which positive results were obtained in each testicular subinoculation, showing that *Treponema pallidum* was present in the popliteal lymph nodes of each rabbit, and second, the infectivity of the treponemata as shown by the rapidity with which the orchitis developed. In eight instances, a testicular lesion was recognized in 29 to 34 days with an average incubation period of 32 days, while in two rabbits, the orchitis developed somewhat more slowly—37 and 45 days. These results are strikingly similar to those obtained from test inoculations of popliteal lymph nodes of latent cases of syphilitic orchitis and are in close approximation to those observed from the inoculation of inguinal lymph nodes of cases with active scrotal lesions.

It might be briefly stated that the three rabbits treated with neoarsphenamine and one of the two treated with arsphenamine subsequently showed an outspoken clinical relapse but that no characteristic lesions developed in the other during a 4 months period of observation following treatment.

The possibility of the recovery of spirochetes from lymph node reservoirs is further emphasized by the results obtained from the continued passage of *Treponema pallidum* from lymph node to testicle. For about 18 months we have carried the Nichols strain of *Treponema pallidum* in eight serial inoculations of inguinal or popliteal lymph nodes to testicles of normal rabbits, thus demonstrating the ability to recover spirochetes from lymphatic tissue through successive generations. With such a procedure, the orchitis tends toward a more prolonged or chronic type with a distinctly longer incubation period which averaged from 4 to 6 weeks. Not infrequently, the reaction consisted in a primary atrophy of the testicle rather than an enlarged granulomatous change. That such a condition is one type of primary response toward *Treponema pallidum* is shown by dark-field demonstration of spirochetes. Moreover, in doubtful cases when no lesions of the testicle can be detected by palpation, infection may be recognized by an adenopathy of the superficial lymph nodes.

Finally in this connection, reference should be made to the use of lymph node reservoirs for preserving stock strains of *Treponema pallidum* when not in active use, thus obviating the necessity of frequent testicular or cutaneous transfers (7).

DISCUSSION.

The studies reported in the present paper were undertaken for the purpose of ascertaining the relation of *Treponema pallidum* to lymphoid tissues in syphilis of the rabbit produced by local inoculation. The problem was approached by a series of experiments designed to show the frequency, time, and extent of lymphatic dissemination of spirochetes from a primary focus of infection in the scrotum or testicle, and the method employed consisted of the demonstration of *Treponema pallidum* in the lymph nodes of infected rabbits by animal inoculation.

The first series of experiments was carried out with emulsions of satellite nodes from rabbits which had been inoculated in the scrotum 2 to 61 days previously. Nodes from twenty-six animals produced a syphilitic orchitis when injected into the testicles of normal rabbits, and spirochetes were found in three others, thus demonstrating that a dissemination of organisms from a primary focus of inoculation constantly occurs by way of the lymphatic system. In only one out of forty-six rabbits inoculated did a progressive orchitis fail to develop, and in this instance, the orchitis was of the atrophic type. In contrast to the high infectivity of the node emulsions is the small number of spirochetes that must have been present in the nodes, since organisms were demonstrated by dark-field examination in only three out of twenty-nine emulsions.

The lymph node emulsions contained little or no microscopic blood, and, in addition, parallel experiments have shown that inoculations of as much as 0.5 cc. of blood rarely produce an infection with an incubation period of 5 weeks.

The second point of importance emphasized by the results of these experiments is in regard to the time of dissemination of the spirochetes from the local focus of inoculation in the scrotum. At the time this work was done, the inguinal lymph nodes showed definite changes, consisting of an enlargement and tenseness or resistance within 4 or 5 days after scrotal inoculation and ultimately progressed to a condition of marked enlargement and induration. With the knowledge that has been gained of the constant dissemination of spirochetes from the scrotum, at least as far as the satellite nodes are concerned, one might logically assume that the early changes in the nodes signified the presence of spirochetes. That this assumption is justified, has been borne out by the consistently positive results of the series of rabbits in which the inguinal nodes were removed 5 and 7 days after scrotal inoculation. The striking feature in regard to the time of dissemination, however, is revealed by the constant infectivity of the inguinal lymph nodes only 2 days after scrotal inoculation, at a time when no gross abnormalities were detected, and, in addition, when no syphilitic reaction about the chancre implant could be recognized. Moreover, it seems justifiable to assume from the evidence at our disposal that enlargement and tenseness of the inguinal lymph node occurring at present as early as 24 hours after scrotal or sheath inoculation are due to the presence of *Treponema pallidum.*

The character or degree of the infection present in the nodes as indicated by the type of orchitis produced by the inoculation of the node emulsions is of considerable interest. There were 57 inoculations made, four of which are omitted from the present analysis (accidental death, etc.). The average incubation period of the orchitis which occurred in the remaining 53 animals determined by the development of palpable lesions was 31 days; in twenty-five animals, it was 29 days or less, and the longest incubation period was 41 days (two animals). Examination of testicular fluid would doubtless have shown the presence of an infection much earlier, but was not carried out on account of the traumatism which may obscure subsequent changes.

It has further been shown that within 48 hours after scrotal inoculation spirochetes have become disseminated and sufficiently established in the animal body to maintain an active progressive infection in the event of the removal of the scrotum with the implant and testicle. Syphilitic lesions of the remaining testicle and epididymis developed in each of the ten rabbits comprising the series in from 40 to 46 days after operation, and, in addition, well marked generalized lesions in each animal were a striking feature of the experiment. These results are not to be interpreted as implying that the inguinal lymph nodes constitute the only focus of infection under the conditions of the experiment, but since it has been shown that spirochetes are constantly present in the inguinal nodes 2 days after scrotal inoculation, it may be assumed that they serve as one such focus. In any event, this experiment demonstrates that the infection itself is so established within 2 days after scrotal inoculation that it is independent of the primary lesion as a source of spirochetes.

The general tendency of *Treponema pallidum* to localize in lymphatic tissue is also shown by the recovery of organisms from other nodes, such as the popliteal and submental. It further appears that such localization is not dependent upon an active lesion in the drainage area of the node but that a widespread dissemination of spirochetes from the local focus of infection occurs by way of the lymphatics. Thus, enlargement and induration of various lymph glands which may occur during the course of a syphilitic infection are significant of the presence of *Treponema pallidum*, and since a popliteal adenitis is a constant feature of syphilis in the rabbit, this condition may be considered as pathognomonic of infection.

The persistence of active spirochetes in the animal body for long periods of time without manifestations other than some abnormality of the popliteal nodes is a fundamental concept of syphilitic infection in the rabbit. We have shown that in cases of true latency of from 3 to 25 months duration, spirochetes may be recovered from the popliteal nodes by animal inoculation. The noteworthy features of this series of experiments were the constancy with which the nodes were shown to harbor active treponemata and the rapidity with which the resulting orchitis developed, the incubation period varying from 31 to 44 days. These results, moreover, are practically the same as those obtained by the testicular inoculation of inguinal lymph nodes from early or active cases of infection.

It would appear, therefore, that although the rabbit possesses some type of mechanism during latent periods of the infection which operates to protect the animal from the harmful toxic effects of the organisms and prevents the development of manifestations of the disease, the organisms themselves are not destroyed but are existent in lymphatic tissue in an active state. Whether other tissues may serve as similar foci for the maintenance of active treponemata for long periods of time is not known, but the well known affinity exhibited by *Treponema pallidum* for lymphatic tissue, together with the clinical evidence of continued adenopathy in latent cases of infection, would point toward this tissue and especially its larger aggregations—the lymphatic nodes—as the principal reservoirs of the virus.

A close similarity existed between the orchitis produced by lymph node inoculation and that caused by an emulsion of either an infected testicle or a skin nodule such as is frequently employed in propagating *Treponema pallidum.* The incubation period of the orchitis produced by inoculation of a node emulsion was about 7 to 10 days longer, but once the process had reached the stage when it could be diagnosed by palpation, its further development proceeded in much the same manner and at about the same speed. This is the more remarkable when the spirochete content of the two inoculating emulsions is considered, spirochetes being numerous in emulsions prepared from a testicular or skin nodule, while it is unusual to see a single organism in those from a lymph node. Thus we are forced to the conclusion by the results of these experiments that actual numbers of spirochetes may play a very small rôle as a causative factor in producing an active progressive syphilitic orchitis and that the state of vitality or degree of infectivity of the spirochetes is of much greater importance.

The ability to recover spirochetes from lymphatic tissue is further emphasized by the results of the serial passage of lymph node emulsion to testicle. During the last 18 months, there have been eight transfers of this type, and in every instance it has been possible to produce a syphilitic orchitis by the inoculation of a lymph node emulsion, the infection of which had in turn resulted from a testicular injection of a lymph node emulsion.

The facts derived from this group of experiments may be profitably utilized in many ways. We have suggested a simple method based upon the duration of lymph node infection for the preservation of stock strains of *Treponema pallidum,* which is both economical and time-saving, and, in addition, is especially effective in guarding against loss of the strain.

A further utilization of the facts derived from these experiments lies in the demonstration of infection in rabbits which show no manifestations of disease as a result of the administration of therapeutic agents. The methods commonly employed in judging the results of therapy have consisted in observations on the effect of the agent upon the spirochetes and upon the regression, healing, and recurrence of the local lesion during observation periods of from 1 to 3 months. In our experience, however, we have seen many instances of a relapse in regions remote from the original lesion with no local recurrence, and, in addition, we know that either a local or a generalized relapse may occur considerably longer after treatment than 3 months. Such rabbits may show a characteristic popliteal adenitis, or there may be no distinctive adenitis, but with the knowledge which has been gained of the persistence of *Treponema pallidum* in lymph node structures, it is possible to demonstrate the presence of spirochetes by animal inoculation without waiting for a clinical relapse. This was done in the case of five rabbits treated with arsphenamine and neoarsphenamine which had shown no local or general manifestations of disease during the 3 months observation period following treatment. The popliteal nodes excised from these rabbits were inoculated into the testicles of normal rabbits with the production of a syphilitic orchitis in all instances. Subsequently four of the five rabbits showed typical local recurrences.

SUMMARY.

A widespread dissemination of *Treponema pallidum* from a local focus of inoculation in the rabbit constantly occurs by way of the lymphatics. Spirochetes were regularly recovered from the satellite lymph nodes by animal inoculation after scrotal inoculation; they were present as early as 2 days, when no specific primary reaction was detected, and at later periods of from 5 to 61 days after inoculation. Other superficial nodes at remote sites such as the popliteals and with no syphilitic lesions in the drainage area have also been shown to harbor active organisms. Although spirochetes were found in relatively few of the lymph node emulsions, the orchitis resulting from their injection was of a rapidly progressive type with an incubation period but slightly longer than that produced by a testicular or skin nodule emulsion rich in spirochetes.

It has further been shown that a syphilitic infection is sufficiently established in the rabbit body within 48 hours after scrotal inoculation so that the primary lesion is no longer essential for its maintenance.

Active treponemata survive in the popliteal lymph nodes for long periods of time and have been regularly recovered from them in cases

of true latency. The lymph nodes, therefore, function as reservoirs of the organisms. The ability to recover the spirochetes from lymphoid tissue through successive generations is seen in the serial passage of lymph node emulsion to testicle during an 18 months period.

The persistence of spirochetes in lymphoid tissue irrespective of the presence or absence of syphilitic lesions is a characteristic and fundamental feature of syphilis of the rabbit. The existence of infection, therefore, may be demonstrated at any time by the recovery of spirochetes from the popliteal lymph nodes by animal inoculation. This fact is of great practical importance in the therapy of the infection and may be profitably utilized in determining the ultimate effect of a therapeutic agent.

These experiments demonstrate that the disease is not confined to the site of local inoculation but that lymphogenous dissemination of treponemata regularly takes place, and that during the course of this process organisms become localized in the lymph nodes and exist there indefinitely irrespective of the occurrence of manifestations of disease. The intimate relation of *Treponema pallidum* to lymphoid tissue is an essential concept of syphilis of the rabbit, and from this point of view, the infection is primarily one of lymphoid tissue.

BIBLIOGRAPHY.

1. Brown, W. H., and Pearce, L., *Arch. Dermatol. and Syphilol.*, 1921, iii, 254.
2. Pearce, L., and Brown, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 164.
3. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1920, xxxi, 709.
4. Brown, W. H., and Pearce, L., *Am. J. Syph.*, 1921, v, 1.
5. Brown, W. H., and Pearce, L., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 201.
6. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1921, xxxiii, 553.
7. Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1921, xxxiv, 185.

STUDIES ON AGGLUTINATION WITH THE AID OF THE CENTRIFUGE. THE INFLUENCE OF TEMPERATURE ON ABSORPTION AND FLOCCULATION.

By FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 13, 1921.)

Whatever opinions may be held regarding the nature of the specific agglutination of bacteria it is generally agreed that the process occurs in two phases; a reaction between cells and serum, in which the antigen and the specific antibodies combine, and a secondary reaction among the affected bacteria in which they cohere and flocculate out of suspension.[1]

Both phases of agglutination proceed gradually to completion and are subject to variation according to the physical conditions under which they occur. Thus, temperature, agitation, mass action, and the influence of electrolytes are known to affect the phenomenon. In the usual agglutination technique the spontaneous flocculation of the bacteria serves as the index of the primary reaction between cells and serum. The complete process, however, includes all the variable factors to which the flocculation phase is subject, and thus the conditions under which the absorption phase proceeds are not open to separate analysis. A method of examination which would eliminate the unknown time element in the flocculation phase, and thus afford a cross-section view of the status of the absorption phase at any period in its progress, might be of value in a closer analysis of some of the factors affecting absorption and perhaps throw some light on the nature of the reaction. The flocculation of bacteria in the presence of their specific antisera may be mechanically effected by means of the centrifuge. The affected organisms, brought into contact by centrifugation, tend to adhere in clumps identical with those produced by the unaided process of agglutination.

---

[1] For the purposes of this paper the current terms absorption and flocculation have been accepted for these phases of the reaction.

Gaehtgens[2] (1906) advocated the use of the centrifuge in agglutination tests with *B. typhosus*. Small, round bottomed tubes of serum and bacterial suspension were centrifuged for 10 minutes without previous incubation, and the resulting sediment was examined macroscopically and microscopically for evidence of clumping. In positive instances a characteristic sediment was formed, which broke up into the typical flocculi of agglutination on shaking, and on microscopic examination showed large clumps of adherent bacilli. The results of centrifuge agglutination with the sera of 100 persons duplicated the findings by the usual method. Control sera, producing no agglutination in the incubator, gave uniformly negative results with the centrifuge. In 1907 and 1908, Brian[3] and Gaehtgens[4] applied the centrifuge method to meningococcus agglutination, which requires 16 to 24 hours incubation at 55°C. by the standard method.

In spite of obvious advantages in point of time and of more exact control over the conditions of test, Gaehtgens' method seems to have attracted little attention. Its failure to find a place among bacteriologic methods may have been due in part to the technical disadvantage of centrifuging the large numbers of tubes often used in agglutination tests, and in part to the confusion which attended the serological diagnosis of the meningococcus—a field in which it might have been useful—before the variations in agglutinin response had been explained by Dopter. Gaehtgens' method seems to have survived for a time only in the diagnosis of glanders, and to have fallen into disuse.

During the period of the war we employed the centrifuge method of agglutination in an extensive series of diagnostic and analytical experiments with the meningococcus group of organisms. In many parallel tests the method was found to be entirely reliable, giving results closely in accord with those obtained by the standard method of incubation at 55°C. for 16 hours.

In the course of these experiments a number of observations were made bearing on certain physical conditions which affect the agglutination reaction. Most of these observations were made on the behavior of meningococci in their specific homologous antisera. These organisms proved to be well adapted for such studies because of the slow rate at which agglutination ordinarily proceeds. The experiments which dealt primarily with the effect of temperature on absorption and flocculation have been collected in this report.

---

[2] Gaehtgens, W., *Münch. med. Woch.*, 1906, liii, 1351.

[3] Brian, O., *Centr. Bakt.*, *1te Abt.*, *Orig.*, 1907, xliii, 745.

[4] Gaehtgens, W., *Arch. Hyg.*, 1908, lxvi, 377.

The Centrifuge Method of Agglutination.

A standardized technique was maintained throughout except as noted in single experiments. The essentials of the method are as follows:

The meningococcus suspensions used were heat-killed cultures which were found to behave in a uniform manner over a period of months.[5] The organisms were grown on glucose agar in Blake bottles for 16 hours at 37°C., washed off with 10 to 20 cc. of sterile 0.8 per cent salt solution, sealed in small bottles, and immediately heated to 65°C. for 30 minutes in a water bath. These concentrated stock suspensions, containing 20 to 50 billion organisms per cc., were stored at 4°C. without a preservative. In some instances the straw-colored supernatant fluid was replaced with sterile saline solution. Dilutions of 2 to 4 billion cocci were made and standardized[6] as required.

Immune serum of rabbits or horses was also stored at 4°C. without a preservative and the successive dilutions were made up fresh for each experiment.

Usually 1 billion organisms were suspended in 1 cc. of dilute serum. The tests were carried out in round bottomed test-tubes, 9 by 90 mm., without lips. They were incubated in a water bath or thermostat, as required by the conditions of the experiment, and centrifuged in groups of five to seven in the wide cups used in the Babcock milk test. An eight cup head on the electric centrifuge thus accommodated 40 to 56 tubes at one time. The specimens were uniformly centrifuged at a speed just sufficient to deposit unagglutinated organisms—10 minutes at 1,800 revolutions per minute.

After centrifugation the tubes were agitated to bring the sediment into suspension and the results read macroscopically by oblique transmitted light against a dark background. Examined in this manner the evidences of agglutination are unmistakable. Unagglutinated organisms, in normal serum or in salt solution, on centrifugation form a compact button in the bowl of the tube. On shaking slightly the sediment comes up in a smooth corkscrew whirl and is evenly distributed throughout the liquid. In the presence of an active serum, however, the central button is surrounded by a fringe of feathery flocculi spread over the bottom of the tube. This flaky layer was well described by Gaehtgens.[2] On shaking, the sediment rises in small clumps or larger masses in a clear or turbid fluid, depending upon the degree of agglutination present. The appearance is identical with that found after spontaneous flocculation. In our experiments three degrees of agglutination were recognized: complete, all the organisms compactly clumped in a clear liquid; incomplete, abundant clumps in a slightly hazy liquid; and partial, small but distinct clumps in a cloudy liquid. In the charts these states are indicated by black bands of different widths, the broad bands indicating complete agglutination. The experiments were carried out with the usual controls in normal serum and salt solution.

---

[5] Amoss, H. L., Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1920, xxxii, 778.

[6] Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

Comparison of Agglutination of Meningococci by the Standard and the Centrifuge Methods.

When Brian and Gaehtgens published their observations nothing was known of the specific variations among meningococci which led to the establishment of types by Dopter and their confirmation by Gordon and by Nicolle, Debains, and Jouan. The first problem, therefore, in the application of the centrifuge method to immunological studies with meningococci was to examine the method for type specificity in comparative tests with the standard agglutination technique.

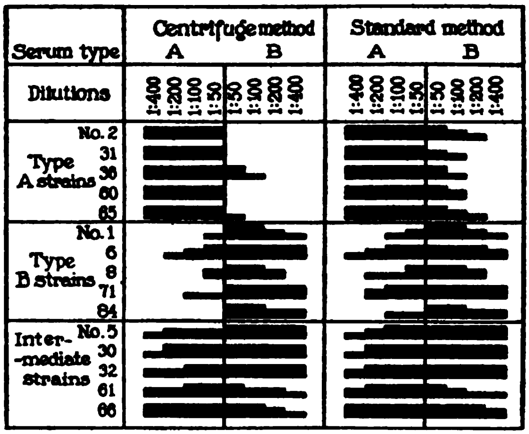
This has been done repeatedly in many experiments with meningococci of spinal or nasal origin, in normal sera and in monovalent or polyvalent antisera from the rabbit or the horse. Typical examples of such comparative tests are shown in Text-fig. 1, for the two main types of meningococci, Types A and B,[7] and for strains which on agglutination show an intermediate character, coming down in almost equal dilutions of the antisera of each type.

Experiment 1.—Type A serum, Horse 33. Type B serum, Horse 32. Sera 1 month old. Serum dilutions 1:50 to 1:400. 1 billion heat-killed organisms suspended in 1 cc. of dilute serum. Duplicate sets. One set centrifuged immediately, shaken, and read. The other set incubated 16 hours at 55°C., shaken, and read. Results in Text-fig. 1.

In this experiment it may be seen that centrifugation without preliminary incubation gives somewhat more clear-cut results in Groups A and B, while in the group of intermediate strains the results by both methods are practically identical. The specific immunological differences among meningococci are thus shown to be discernible by this method, and indeed are accentuated rather than decreased, so that the centrifuge method is, perhaps, the more useful for type diagnosis. These differences are probably due in part to the fact that the so called coagglutinins seem to react more slowly than do the specific agglutinins and require a longer incubation time for their demonstration. A discussion of this interesting difference is not within the scope of this paper.

---

[7] Following Dopter we accept the classification of Nicolle, Debains, and Jouan, in which the meningococcus is designated Type A, and Dopter's parameningococci, alpha, beta, and gamma, are called Types B, C, and D respectively.



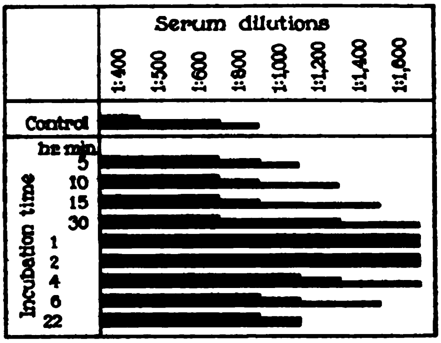
TEXT-FIG. 1. Centrifuge agglutination of meningococci without incubation *versus* the standard method, incubated 16 hours at 55°C.

The Effect of Incubation at 55°C. on the Serum Titer.

In general, centrifuge agglutination without incubation has given results of somewhat lower titer than has spontaneous agglutination after 16 hours incubation at 55°C. This fact led us to combine a short incubation period with the mechanical flocculation, and in this way the highest titers with a given serum were obtained. These observations were based on experiments such as the following.

Experiment 2.—Type B meningococcus, Strain 1, agglutinated in homologous monovalent antiserum from a rabbit. Suspensions of the killed culture, in successive dilutions of the antiserum, were centrifuged without incubation to determine the approximate limits of agglutination. Beginning with serum dilutions near the upper limit of complete agglutination, coccus-antiserum mixtures were set up at close dilution intervals, incubated at 55°C. for periods of 5 minutes to 22 hours, centrifuged, shaken, and read. The results are given in Text-fig. 2.

A period of incubation promotes absorption to such an extent that complete clumping occurs in dilutions two to four times higher than without previous incubation. Even 5 minutes exposure to 55°C. has an obvious effect. The highest serum titer is found after 1 to 4 hours incubation. Long exposure, 16 to 24 hours, to 55°C. may cause a diminution in the titer. This observation has been made repeatedly and is due to a deleterious effect of the high temperature on the serum antibodies, as will be shown presently. Repetitions of Experiment 2 with other strains and sera have given practically identical results.



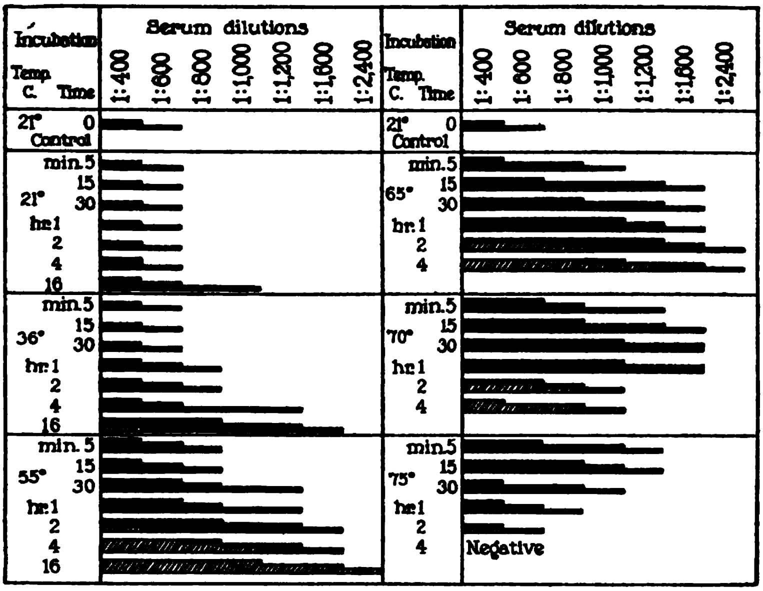
Text-Fig. 2. The effect of incubation at 55°C. on the titer of agglutination in antimeningococcus serum.

The Effect of Temperature on Absorption.

The foregoing experiment shows that contact of organisms and antisera for 1 to 4 hours promotes a reaction sufficient to produce clumping on centrifugation in high dilutions of the antiserum. Is the temperature at which the reaction takes place a function of the time required for its completion?

Experiment 3.—Suspensions of a Type B organism, Strain 1, were incubated at various temperatures and for varying periods of time in successive dilutions of a homologous monovalent antiserum from a rabbit. After the determined incubation period (water baths) the tubes were read to note the occurrence of spontaneous flocculation, and were then centrifuged, shaken, and read. The results are given in Text-fig. 3.

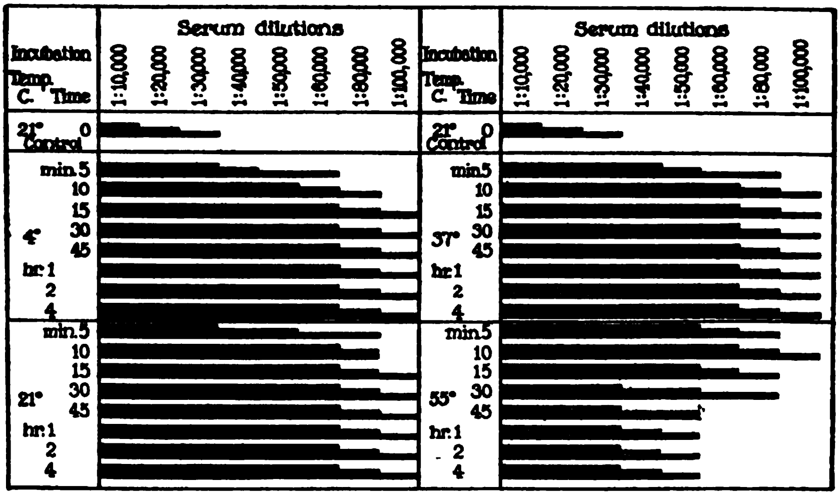
In the preliminary test, without incubation, complete agglutination occurred at 1:200 (not shown on the chart) with the limit of visible clumping at 1:600. In these serum dilutions the bacteria absorbed enough agglutinin during their passage to the bottom of the tube to cause them to cohere in spite of resuspension. This reaction is a constant in each experiment and forms the base-line in each test. No more absorption than this occurred at 21°C. after 4 hours contact, but after 16 hours some agglutination was found in a dilution of 1:1,000. At 36° absorption proceeded more rapidly and a positive reaction was evident after a contact of 1 hour. As already noted in Experiment 2, 5 minutes contact at 55° promotes absorption, which, however, did not reach the limit, 1:2,400, until more than 4 hours had elapsed. At the higher temperatures the effect of heat is more quickly apparent, so that at 65° the limit of the reaction was reached in 2 hours and at 70° and 75° the maximum absorption occurred at 30 and 5 minutes respectively. At these high temperatures the serum antibodies were already in process of destruction so that the titer fell off rapidly as incubation was prolonged. Similar



TEXT-FIG. 3. The influence of temperature on the absorption of meningococcus agglutinins. Spontaneous flocculation cross-hatched; centrifuge agglutination in black.

results were obtained in a parallel experiment with a Type A meningococcus in its antiserum.

Although the agglutination reaction is not well adapted to accurate quantitative estimation because the end-point is not sharp, it is interesting to note in these experiments that the time and temperature relations are roughly those of chemical acceleration. The behavior of the serum is also similar to that of enzymes, which are most active just below the temperature of rapid destruction.



TEXT-FIG. 4. The influence of temperature on the absorption of typhoid bacillus agglutinins. Spontaneous flocculation cross-hatched; centrifuge agglutination in black.

It is only with an organism such as the meningococcus which reacts with its antiserum at a measurable rate that such an experiment can readily be performed. *Bacillus typhosus* reacts with its specific antiserum so rapidly that the process seems to be complete in 15 minutes at 4°C. and in 10 minutes at 36°C.

Experiment 4.—Suspensions of heat-killed typhoid bacilli in high dilutions of antiserum from a horse. Identical sets of tubes incubated at various temperatures for varying periods of time. Read to note spontaneous flocculation, centrifuged, shaken, and read. The results are given in Text-fig. 4.

High temperature quickly showed an injurious effect on the *Bacillus typhosus* agglutinins. The titer of the serum began to fall off after an exposure of 10 minutes at 55°C.

The Effect of Temperature on Flocculation.

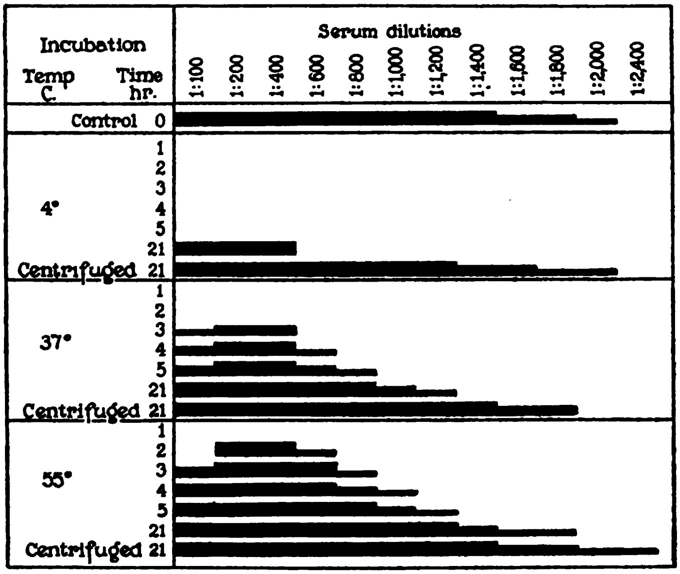
From the readings of spontaneous flocculation obtained in Experiments 3 and 4 before centrifugation of the specimens it was found that the higher temperatures accelerate the flocculation phase of the agglutination reaction also. These readings are indicated in Text-figs. 3 and 4 by cross-hatched areas within the solid black bands.

Although the centrifugation of the incubated specimens of Experiment 3 shows that some absorption had occurred in 16 hours at 21°C. and even in 1 hour at 36°, practically no unaided flocculation resulted at these temperatures in the serum dilutions at which the tests were made. At higher temperatures flocculation appeared earlier and in higher dilutions, but in no instance did it become visible in less than 2 hours, even though the absorption phase had reached its limit in 15 to 30 minutes. Most of the incubation time involved in spontaneous agglutination is required by the relatively slow development of visible flocculation. The influence of temperature upon flocculation is more strikingly shown in the experiment with *Bacillus typhosus*. Here absorption was complete in 15 minutes at 4°C. At this temperature flocculation was not evident for over 2 hours, whereas at 37°C. it appeared in 30 minutes in the lowest dilution examined, and at 55° it appeared in 15 minutes.

In the following experiment with a Type B meningococcus and its homologous antiserum absorption was completed in a short time by exposure to a high temperature and the subsequent flocculation allowed to proceed at various temperatures to determine the relative rate of its development.

Experiment 5.—Type B meningococcus, Strain 1. Homologous monovalent antiserum from a rabbit. Four sets of killed culture suspensions in successive serum dilutions. Heated at 70°C. for 30 minutes. One set centrifuged immediately as a control to determine the completion of the absorption phase. Other sets placed at 4°, 37°, and 55°, and examined at intervals. After 21 hours all the sets were centrifuged to confirm the completion of absorption. The results are given in Text-fig. 5.

These observations on the time and temperature relationships of the flocculation phase reveal the handicap that attends an attempt to study the specific phase of the agglutination reaction with spontaneous flocculation as the indicator. An analysis of these experiments shows that even at high temperatures flocculation does not become visible until long after absorption has progressed to the point required for complete agglutination, while at low temperatures, in high dilutions of the antiserum, flocculation may not appear at all, even though absorption has advanced appreciably. In order to produce a visible result, spontaneous flocculation requires an absorption reaction several times in excess of that required by the centrifuge method of agglutination.

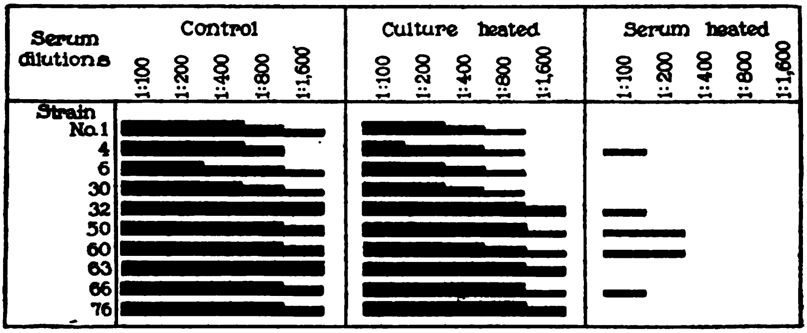


TEXT-FIG. 5. The influence of temperature on meningococcus flocculation.

The Destructive Effect of High Temperatures on Meningococcus Agglutinins.

The decline in agglutination titer which results from prolonged incubation at high temperatures is illustrated in the foregoing experiments. Such a destructive effect was extensively studied by Eisenberg and Volk (1902), by Joos (1903), by Kraus and Joachim (1904), and by Scheller (1904–05). The following experiment, showing that this deleterious effect is exerted on the serum antibodies rather than on the bacteria, is cited to show the application of the centrifuge method to problems of this character.

Experiment 6.—Year old polyvalent antimeningococcus serum, in successive dilutions, and killed culture suspensions of a number of meningococcus strains, 2 billion per cc., were each divided into three lots and one lot of each was heated at 70°C. for 30 minutes, an incubation period found to have an injurious effect in agglutination tests with these materials. Tests were then set up with mixtures of (*a*) unheated serum and unheated organisms (control); (*b*) unheated serum and heated organisms; and (*c*) heated serum and unheated organisms. These tests were incubated at 55°C. for 4 hours, centrifuged, shaken, and read, with the results shown in Text-fig. 6.



TEXT-FIG. 6. The destructive effect of high temperatures on agglutinins.

It is seen that the unheated serum and heated cocci react in a manner similar to the controls, whereas the heated serum has lost much of its agglutinating activity. Experiments already cited show that the antigen-antibody complex, after the specific reaction has occurred, is still subject to injury by high temperatures. In Experiment 3, for example, the decline in the agglutination limit on prolonged exposure to 65°, 70°, or 75°C. occurred after the absorption reaction was complete at these temperatures. Tests with antisera which have been stored for some time show that they are particularly subject to the effects of high temperatures. The typhoid serum used in Experiment 4 was 3 years old, which may account for the decline in the agglutinin titer even at 55°C.

The Relation of Temperature to Mass Action.

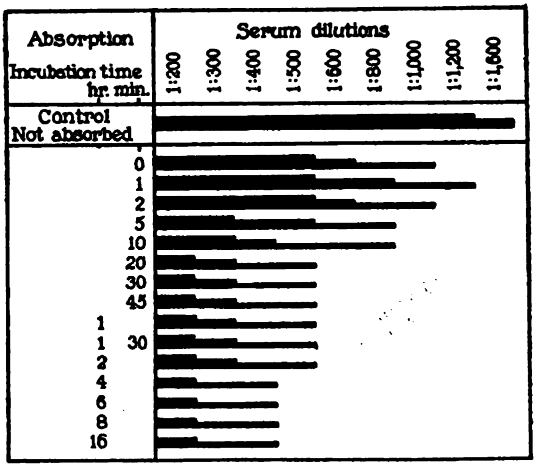
A survey of Text-fig. 3 shows that 0.001 cc. of the antiserum in a volume of 1 cc., a dilution of 1:1,000, contained enough agglutinins completely to clump 1 billion homologous organisms. This complete reaction required more than 4 hours for its development at 55°, but developed in 1 hour at 65° and in 30 minutes at 75°. In lower dilutions, containing an excess of antibodies, a reaction sufficient to clump all the organisms occurred much earlier at these temperatures, and even at 36° and 21° it occurred in the 1:400 dilution in 1 and 4 hours respectively. The presence of an excess of agglutinins greatly accelerates absorption. This fact has long been known, as has the fact that a given bacterial suspension may remove from an excess of serum several times the amount of agglutinins required for its complete flocculation (Eisenberg and Volk[8]). It is interesting to note, however, with the aid of the centrifuge, how quickly the process is brought to its conclusion.

Experiment 7.—Tubes containing 4 billion Type A meningococci, Strain 60, in 2 cc. of homologous antiserum diluted 1:100, were incubated at 55°C. for various periods of time. After centrifugation the clear, supernatant fluids were decanted and diluted successively, mixed with fresh lots of cocci (1 billion organisms in 1 cc. of serum), incubated at 55° for 4 hours, centrifuged, shaken, and read. The results are given in Text-fig. 7.

From the results of the control agglutination it is seen that the serum in a dilution of 1:1,200 originally contained enough antibodies completely to agglutinate 1 billion meningococci, a quantity which may be designated as 1 unit of agglutinin. Before absorption, therefore, each 2 cc. specimen, 1:100, contained 24 agglutinin units. The mere act of centrifugation, without incubation, in contact with 4 billion organisms, deprived the serum of about 12 units of agglutinin, leaving it with a titer of 1:500 to 1:600, so that each billion meningococci removed about 3 agglutinin units. 1 or 2 minutes incubation did not increase absorption appreciably, but during 5 minutes contact at 55° the serum titer dropped to 1:300, a loss of 18 units, and during 20 minutes at 55° the titer fell to 1:200, indicating the absorption of about 20 units. Each billion meningococci had absorbed about five times the agglutinins necessary for their complete flocculation. This appears to have been about the absorption limit, at this serum dilution, for after 20 minutes only a few more agglutinins were removed from the serum. In other experiments the reaction came to a standstill after 30 and 90 minutes respectively. It is evident that the incubation of absorption tests

---

[8] Eisenberg, P., and Volk, R., *Z. Hyg. u. Infectionskrankh.*, 1902, xl, 155.



TEXT-FIG. 7. Agglutinins left in antimeningococcus serum after absorption at 55°C. with the homologous organisms for various periods of time.

for long periods of time does not result in the removal of correspondingly large quantities of agglutinin. Repeated absorption with fresh bacteria over short incubation periods is the more effective method of removing specific antibodies from serum.

SUMMARY.

The flocculation of bacteria which have absorbed specific agglutinins may be mechanically effected by means of the centrifuge, with results that coincide with those obtained by the standard method of test. Specific serological differences between meningococci, for example, may be determined by the centrifuge method. The technique is described.

By the elimination of the inconstant time factor in the flocculation phase opportunity is given for a closer analysis of specific absorption, and of the influence of various conditions upon both phases of agglutination.

The velocity of the absorption reaction is a function of the temperature at which it occurs, and the acceleration with increasing temperature is of the order of chemical phenomena. The absorption reaction proceeds most rapidly near the temperature of antibody destruction. The injurious effect of high temperature is revealed first in the serum; the antigen-antibody complex is not less sensitive.

The flocculation phase is also promoted by higher temperature, but lags far behind absorption, and consumes most of the time required for spontaneous agglutination.

The presence of an excess of antibodies greatly accelerates absorption and flocculation. The absorption reaction, under such circumstances, is ordinarily completed within a relatively short time.

THE EFFECT OF PULMONARY CONGESTION ON THE VENTILATION OF THE LUNGS.

By CECIL K. DRINKER, M.D., FRANCIS W. PEABODY, M.D., and HERRMANN L. BLUMGART, M.D.

(*From the Laboratory of Applied Physiology of Harvard Medical School and the Medical Clinic of the Peter Bent Brigham Hospital, Boston.*)

Plates 1 to 3.

(Received for publication, June 21, 1921.)

Clinical observations have shown that in patients with heart disease the vital capacity of the lungs is frequently less than normal, and that the decrease in the vital capacity bears a close relation to the development of dyspnea. As the tendency to dyspnea increases the vital capacity usually falls, and in patients who are bedridden on account of shortness of breath the vital capacity is rarely more than 30 per cent of the normal. This lowering of the vital capacity expresses the impossibility of increasing the depth of respiration in a normal manner, and under the stress of exercise such subjects are unable to raise the volume of pulmonary ventilation so as to bring about the necessary gas exchange between lungs and blood. The determination of the vital capacity has, therefore, considerable practical significance, since it serves as a guide to the general condition of the patient and often indicates changes in the functional condition of the circulation and respiration that are in harmony with the symptoms but are not necessarily suggested by changes in the physical signs.

The cause of this decrease in the vital capacity of the lungs in heart disease has never been adequately explained. In advanced cases it is, of course, due in part to pulmonary edema, pleural effusion, hepatic enlargement, and similar obvious factors; but in many cases the vital capacity is decreased without any physical signs which account for it, or with physical signs which are certainly insufficient to explain the degree of decrease.

The suggestion has been made (1, 2) that in such cases there may be an increase of pressure in the pulmonary circulation with engorgement of the alveolar capillaries of the lungs. As the alveoli are extremely vascular such a condition might produce a stiffening or *Lungenstarrheit*, in the sense of von Basch (3), which would interfere with their easy expansion and collapse in respiration. There might also be some protrusion of the distended capillaries into the alveoli and thus a decrease in the volume of residual air of the lungs. Since it is impossible to measure pulmonary arterial pressure in human beings, no statements can be made as to the degree of pressure increase which may develop in cardiac disease. From our observations upon cats in which the pulmonary veins have been obstructed, it is apparent that a high degree of pulmonary stasis may be produced without causing much actual increase in pulmonary blood pressure. The lungs act as a slightly elastic sponge, and are able to take up a vast amount of blood without significant pressure change. Furthermore, the normal right ventricle is unable to sustain pressures of any magnitude. We have attempted to increase pressure in the pulmonary artery through a clamp placed upon this vessel just central to its division. It is impossible to adjust such a clamp so as to provide a sustained increase in pressure of more than a few millimeters of mercury in the right ventricle of the cat without causing the death of the animal. We may, therefore, consider that when blood accumulates in the pulmonary circuit with a normal heart there is little attendant pressure increase, and that accompanying changes in ventilation are incident upon volume rather than pressure changes in this circulation.

In chronic cardiac disease with hypertrophy of the right ventricle it is conceivable that pressure in the pulmonary capillaries may reach abnormal levels, but it should be remembered that at the time we are interested in the question—the moment when the vital capacity begins to diminish—the heart muscle is in all probability beginning to fail, and, as a consequence, we are inclined to believe that even in human beings with considerable cardiac hypertrophy the rise in pressure in the pulmonary circuit accompanying a much increased blood volume in this circuit is not great. We are at present engaged upon experiments in which the pressure changes in the pulmonary artery and capillaries resulting from pulmonary vein compression are being followed, and the statements just made must, therefore, be taken as expressions of our opinion at the moment, not as facts of experimental observation.

Clinical experience contributes several facts which are in harmony with the theory that the interference with the ventilation of the lungs, which shows itself by a decrease in the vital capacity, is due to a chronically increased filling of the pulmonary veins and capillaries. Thus, mitral stenosis is characterized by an early onset of the tendency to dyspnea and an associated low vital capacity. In aortic insufficiency, on the other hand, there is little tendency to dyspnea, and the vital capacity remains high until a relative mitral insufficiency develops and the pulmonary circulation is affected. Again, pleural effusions and pulmonary edema with râles are accepted signs of cardiac weakness. These probably find their cause in congestion of the pulmonary capillaries, and it is logical to suppose that their appearance is preceded by a phase in which the pressure may be very slightly higher than normal, but not yet sufficient to bring about exudation into the alveoli with eventual production of râles or the passage of fluid into the pleural cavity. Before either of these processes takes place there are no physical signs to indicate definitely changes in the pulmonary blood circuit. According to the theory suggested, however, even at this stage the vascular engorgement might interfere with the movements of the lungs and with the size of the alveoli, and cause a decrease in the vital capacity. If this theory is true, its practical significance will be easily understood, for the determination of the vital capacity will give the earliest and most accurate information which can be obtained about the pulmonary circulation. The earliest symptom in most cases of heart disease is an increased tendency to dyspnea and throughout the course of the disease this tendency is usually one of the best guides to the functional efficiency of the heart. It seems quite possible that the development of dyspnea depends largely on the condition of the circulation in the lungs, and, if such be the fact, any method which will help to throw light on one of the most obscure portions of the circulation will be of great clinical importance.

The problem which presents itself, therefore, as the result of the foregoing clinical observations, is to determine whether blood stasis in the pulmonary circulation produces any effect on the ventilation of the lungs. If such a change does produce a limitation or reduction of the ventilation, then the theory that the reduction of the vital capacity of the lungs in heart disease is due to an increased filling of the pulmonary circulation receives considerable support. It is obvious that the question cannot be decided by the study of patients; the animal experiments to be described in the present communication were, therefore, designed in order to elucidate it.

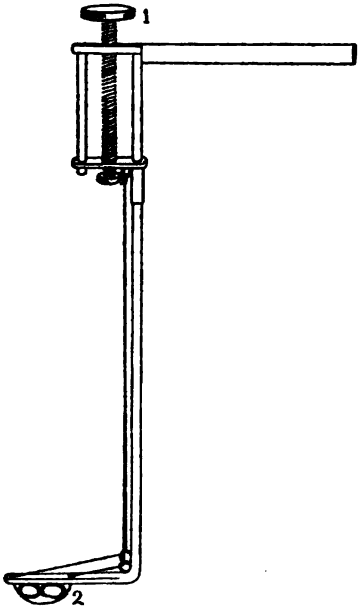
Technique.

The animals selected for study were cats. Because of the exquisite delicacy of the tissues to be studied and the relatively slight displacement in air space to be measured, certain experimental requirements were considered essential.

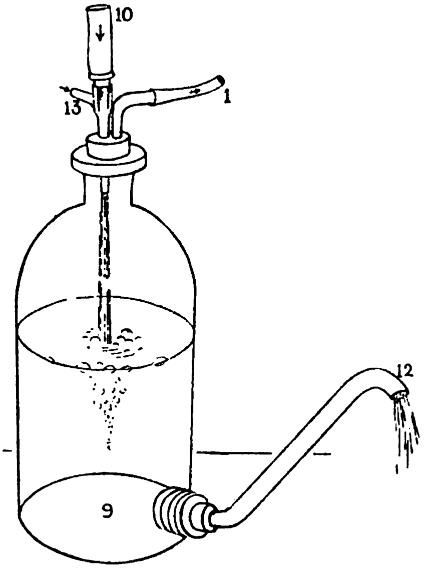
1. All spontaneous and reflex movements of the animal had to be eliminated. Even in the anesthetized animal compression of the pulmonary veins brings about active respiration, and such a reaction interferes with comparative measurements. Urethane anesthesia followed by curare obviates this difficulty.

2. A method had to be devised for compressing and releasing the pulmonary veins without any manipulation of the lungs. This was satisfactorily accomplished by the method described in detail previously (4). An oval section of the chest wall immediately over the heart is removed, the pericardium incised anteriorly, and the cut margins are reflected and sewed to the edges of the chest wall. With proper technique, the pleural cavity is rendered air-tight, permitting for hours independent respiration of the animal. With such a method the heart and great vessels lie exposed *in situ*, resting posteriorly on a pericardial sling. Advantage is then taken of the unusually high reflection of the pericardium in the cat, in which animal a ligature may be slipped about the pulmonary veins without in any way entering the mediastinum. The ligature is then threaded into the clamp as indicated in Text-fig. 1. The pulmonary veins, 2, represented in cross-section, can then be compressed and released by manipulation of thumb-screw 1. We have also found it possible to insert into the pulmonary artery a cannula of the type described by Schafer (5), in order to measure pressure in this artery during pulmonary vein compression.

All of these adjustments can be made after closing the thorax with the pericardium without the use of artificial respiration. In certain instances, of which Experiment 4 is an example, an intrathoracic cannula was inserted and connected either with a water manometer or with a delicately balanced volume recorder. Under such circumstances intrathoracic pressure or lung volume may be followed and the influence of blood in filling up thoracic space noted with accuracy.



Text-Fig. 1.



Text-Fig. 2.

Text-Fig. 1. Clamp for compression of pulmonary veins.
Text-Fig. 2. Apparatus for obtaining constant air pressure.

But in these instances the lungs are invariably collapsed away from the chest wall and are thus able to swell outwardly when they fill with blood. Such a condition is not permitted when the chest wall is properly closed and the lungs are well inflated against it.

3. The air delivered to the animal had to be at a relatively constant pressure and uniform rate of flow. This condition was satisfied by the apparatus illustrated in Text-fig. 2. The water flows down through tubing 10, under a constant head which is maintained by

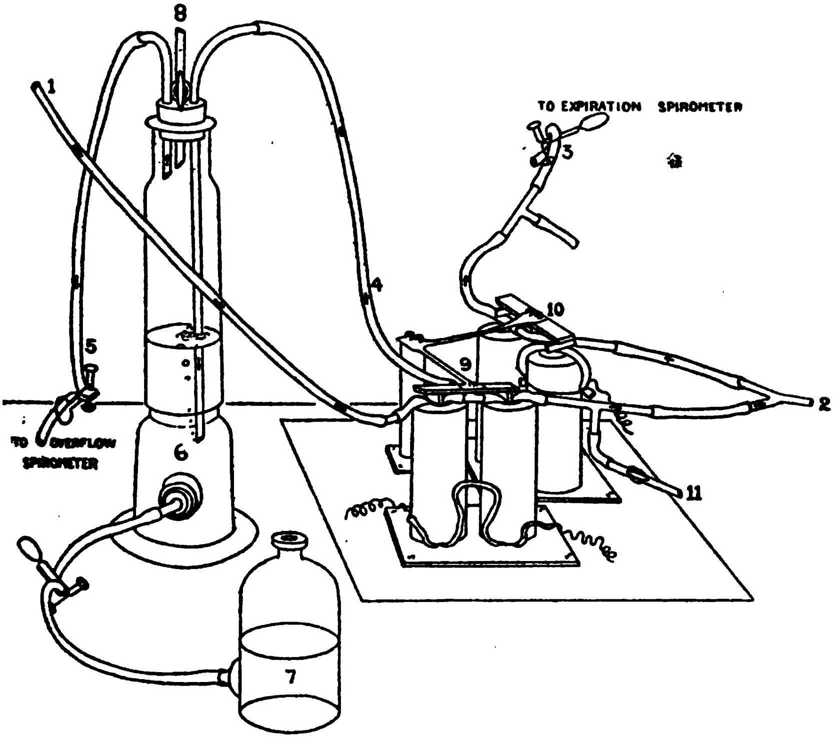
a bottle suspended 9 feet above, kept full to overflowing. The water passes through an ordinary suction pump, draws in air through bypass 13, and runs into an air-tight bottle, 9. Here air and water separate. Since the water level in the bottle is determined by the height of the top of the overflow tube, 12, the added amount of water passes out through this overflow tube, while the air is conducted out through tubing 1 to the animal.

4. The apparatus had to be so designed as to register quantitatively any alteration in pulmonary ventilation. The artificial respiration machine indicated in Text-fig. 3 fulfills this need. It consists of two magnetic interrupters timed by a rotary switch to compress alternately the rubber tubing beneath them. The air passes to the animal from the bottle shown in Text-fig. 2 through an inspiratory tube, 1 (Text-fig. 3), to the cannula, 2, inserted into the trachea of the cat. During the period which constitutes inspiration, one hammer, 9, is raised, while a second hammer, 10, completely occludes the expiratory tube, 3. During expiration, the reverse situation exists, the expiratory air delivered through tube 3 being collected in a sensitive spirometer, known hereafter as the expiration spirometer.

Attention should be drawn to the shunt interposed between the two bars of hammer 9. This shunt, 4, by virtue of its position, operates only during inspiration and is designed to conduct all the air not accommodated by the cat's lungs through bottle 6 to another finely balanced spirometer, termed the overflow spirometer. If the available air space in the lungs is diminished, or the elasticity of the lungs becomes decreased so that they no longer expand as much under the same air pressure, more air will be collected in this overflow spirometer while less air will be collected in the expiration spirometer.

The construction and purpose of bottle 6 interposed in the overflow circuit deserve further comment. The air flows in through tubing 4, bubbles through the water in bottle 6, and escapes to the room through a stop-cock, 8, or, if this be closed and the clamp released, through tubing 5 to the overflow spirometer. Regardless of the avenue of escape of this air, however, the height of the water column in bottle 6, adjustable through container 7, determines the amount of air delivered to the animal. Thus, if the water column is high, little or no air will escape during inspiration into the overflow spirometer and all the air will pass into the animal to be delivered to the expiration spirometer.

In brief, provision is made to collect all the air delivered to an anesthetized, curarized animal. This air reaches the animal rhythmically but under relatively constant conditions of pressure and flow.



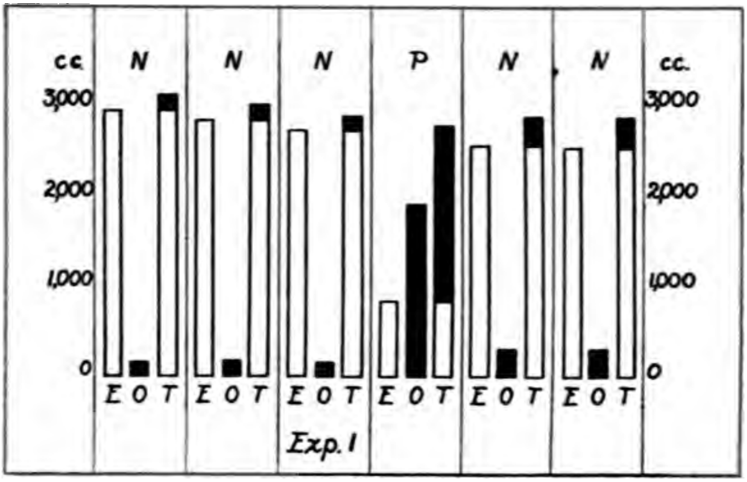
TEXT-FIG. 3. Magnetic interrupter for artificial respiration, with connections to spirometers.

It is collected in two spirometers and the total amount of air which these two receive will check closely in different periods of equal length. But if the pulmonary air space is decreased or if the movements of the lung tissue are restricted, the distribution of the collected air will at once change, a smaller amount being received by the expiration spirometer, and a larger amount by the overflow spirometer.

EXPERIMENTAL.

Experiment 1.—February 7, 1921. Cat; weight 2.8 kilos. 12.05 p.m. 28 cc. of 25 per cent urethane by stomach tube. 3.05 p.m. Operation on thorax completed. 3.30 p.m. 1 cc. of 2 per cent curare intravenously. Cardiometer and pulmonary vein clamp adjusted. Arrangements made for recording rate of respiration and femoral blood pressure.

The further course of this experiment is illustrated by Text-fig. 4 and Fig 1. Text-fig. 4 is a graphic representation of the six collection periods which comprised the experiment. There are, first, three normal periods, labeled *N*, in which air delivered to the animal was collected in the expiration and overflow spirometers without any obstruction of the pulmonary circulation; then one period, labeled *P*, in which the pulmonary veins were compressed by means of the clamp shown in Text-fig. 1; and finally, two more periods, labeled *N*, prior to which the pulmonary vein clamp was released and the animal permitted to return to normal conditions. All periods in this and other experiments were 5 minutes in length, and in Text-figs. 4 to 7 the same method of representation is used. In every case the column marked *E* is the amount of air which actually entered the lungs of the cat and was collected in the expiration spirometer; the column marked *O* is the amount received in the overflow spirometer; and the column marked *T* is the



TEXT-FIG. 4. Graphic representation of Experiment 1. Six 5 minute periods are shown. In this and the following text-figures *N* indicates normal period; *P*, period of pulmonary vein compression; *E*, air collected in expiration spirometer; *O*, air collected in overflow spirometer; *T*, total air collected by both spirometers.

total amount of air delivered by the respiration apparatus during the 5 minute period, a total obtained by superposing the overflow column upon the expiration column.

Fig. 1 is a kymograph record of conditions during parts of the experiment. Section 2 covers the second period charted in Text-fig. 4. Tracing 1 is the cardiometer record made with the usual type of cardiometer transmitting through air to a large tambour. Tracing 2 is made by means of a second, rather stiff tambour and indicates the rate of respiration. A rubber tube (11, Text-fig. 3) on the inspiratory side of the respiration apparatus leads to this second tambour. Tracing 3 is the blood pressure record taken from the femoral artery by means of a mercury manometer. Tracing 4 is the base-line for blood pressure. Downward marks upon this line indicate 15 second intervals, but in this case are too irregular to be of value since the time-clock was out of order. Upward marks signal various events in the experiment.

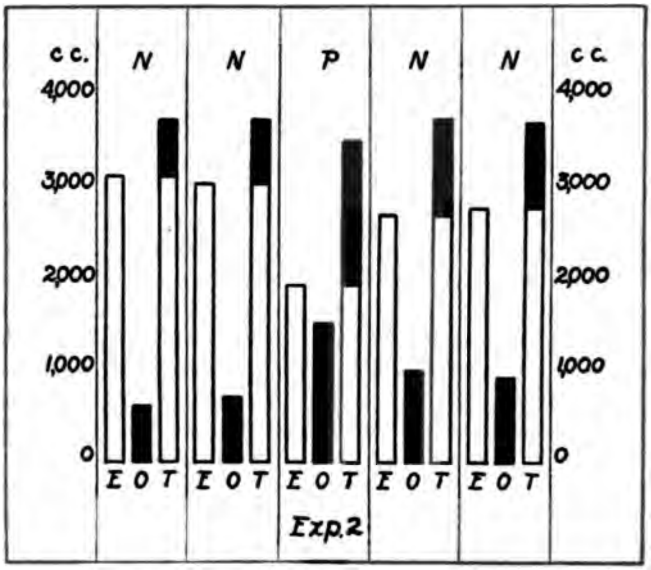
During the first three normal periods, a constant amount of air was collected in the overflow spirometer but the total amount was slightly reduced. The change was, however, so much less than that occurring when pressure was applied to the pulmonary veins as to be negligible.

The first upward mark on the base-line in section 4, Fig. 1, indicates the beginning of pulmonary vein compression, brought about by tightening the clamp shown in Text-fig. 1. Between the second and third upward marks in this section of tracing, the air collection shown in *P*, Text-fig. 4, was made, and at the fourth upward mark the pulmonary vein pressure was released. The rise in the cardiometer record during this period shows a certain amount of cardiac dilatation; and the diminished movements of the writing point, a restriction of the individual beats. The fall in blood pressure indicates the degree of interference with filling of the left ventricle. The shift in distribution of the air during this period is clearly shown in *P*, Text-fig. 4. The great reduction in the amount of air collected in the expiration spirometer was due to pulmonary congestion incident upon the pulmonary venous obstruction, and the rise in the overflow collection merely accounts for the air which could not enter the lungs under the conditions imposed.

The two last normal periods *N* and *N*, Text-fig. 4, indicate an almost complete recovery after pulmonary vein release. The fact that this was not absolutely complete probably indicates a certain amount of intraalveolar extravasation which was not at once removed and which occupied space available for air in the first three normal periods. It is, however, clear that a very large measure of recovery did take place, and this means that the changed distribution of air noted in period *P*, Text-fig. 4, was due to intravascular blood.

The lungs, when removed at the close of this experiment, showed narrow longitudinal areas of congestion along the most posterior and consequently dependent portions of each lobe. The degree of change was comparable to that shown in Fig. 5, taken, it is true, from another experiment, but fairly indicative of the pathological changes occurring in experiments accepted by us as picturing the true relation of vascular filling and ventilation. On microscopic examination one finds epithelial desquamation with red cells and fluid in the alveoli making up the narrow area commented upon in the gross description of the lungs, and normal appearances in the rest of the tissue.

Experiment 2.—February 19, 1921. Cat; weight 3 kilos. 30 cc. of 25 per cent urethane. Operation and preparations for recording similar to those in previous experiment. Text-fig. 5 shows two normal periods, then a period of pulmonary vein compression, and finally, two more normal periods. The result is identical with that noted in Experiment 1. Fig. 2 is a graphic record similar to that in Fig. 1. Section 1 corresponds with the first normal period. Since pulmonary vein compression was produced rather slowly, section 3 has been shortened to save space. The first upward mark on the base-line indicates the beginning of compression. Between the second and third, the air collection noted in *P*, Text-fig. 5, was made, and at the fourth mark the veins were released. Section 4 corresponds with the first normal period *N* after *P* in Text-fig. 5, and displays the condition of the circulation at that time.

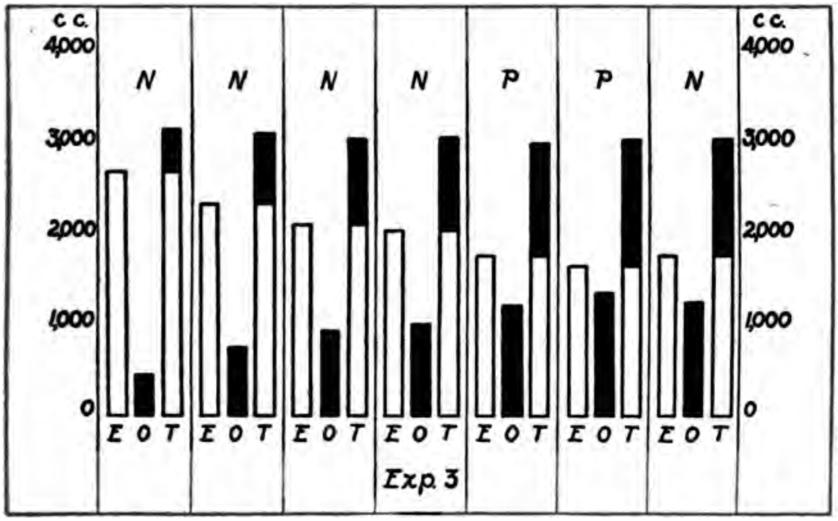


TEXT-FIG. 5. Graphic representation of Experiment 2. Five 5 minute periods are shown.

On examination of the lungs an area of dark red congestion—not hard nor raised—was noted along the posterior surface of the right lung, and a few similar very small congested areas, chiefly along the margins of the lungs. It is interesting to note that the amount of overflow air in the last collection was somewhat less than in that just preceding, indicating a gradual tendency to return to normal.

In this case, the pulmonary vein clamp was tightened at 12.47 p.m., and collection period *P* took place between 12.56^{30} and 1.01^{30} p.m. The first normal collection period after clamping fell between 1.11^{10} and 1.16^{10} p.m. and the last one between 1.23 and 1.28 p.m.

Experiment 3.—February 19, 1921. Cat; weight 2.9 kilos. 29 cc. of 25 per cent urethane by stomach tube. A technique exactly similar to that of the two preceding experiments was used in this case except that two periods of pulmonary vein compression (*P*, *P*, Text-fig. 6) were employed. The result shows the effect of a progressively weakening heart and the production of extensive pulmonary edema. The heart was unusually large at the beginning of the experiment and there was more pericardial fluid than usual. Four normal periods were run and the volumes of air collected in the overflow spirometer showed an increase from 473 in the first period to 1,023 cc. in the fourth period. This increase was probably due to the development of pulmonary edema. In the third and fourth normal periods the volume in the overflow spirometer became more constant than it was in the first two, so that light compression of the pulmonary veins was tried in the 5th period.



TEXT-FIG. 6. Graphic representation of Experiment 3. Seven 5 minute periods are shown, two of them in this instance being periods of vein compression.

The condition of the animal at this time is shown in section 5, Fig. 3. Poor adjustment of the cardiometer with leakage explains the atypical cardiometer record. Light compression caused a characteristic effect as noted in the first *P*, Text-fig. 6, and was followed in 9 minutes by a period of greater compression, the second *P* in Text-fig. 6, and section 6 in Fig. 3. A greater degree of the typical change occurred, and that this was due in part to vascular engorgement, and not wholly to the progressive development of pulmonary edema, is indicated by the fact that release of the vein compression resulted in the last normal period (Text-fig. 6) in a slight return towards normal conditions. Section 7 in Fig. 3 indicates the condition of the animal at this time—a condition of progressively dilating heart and falling arterial pressure, the full extent of which was obscured by a clot which occurred near the end of the tracing.

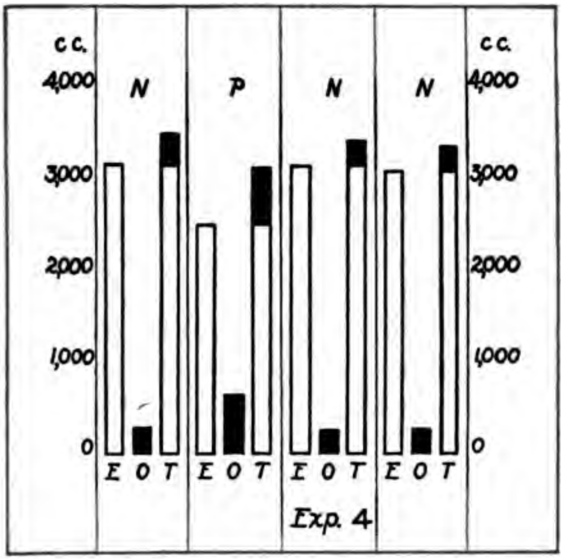
The lungs at the termination of this experiment were in bad condition, over two-thirds of the posterior surface being congested and dark red. There was, thus, extensive permanent damage. The experiment is published in order to display the course of events when pulmonary air space is steadily encroached upon by something more than intravascular blood. Such an animal is perhaps in a similar condition to that of the patient with reduced vital capacity and clear-cut physical signs in the lungs.

Experiment 4.—May 2, 1921. Cat; weight 2.7 kilos. 27 cc. of 25 per cent urethane by stomach tube. All preparations were exactly the same as in preceding experiments with one exception. The line of tracing numbered 2 in Fig. 4 indicates in this instance, intrathoracic volume changes. With the preparation completed in other respects, a glass tube was thrust through the chest wall and tied in place by a purse-string suture. Lung collapse at once occurred, probably bilateral. The intrathoracic cannula was connected with a delicate volume recorder constructed to write on a kymograph. On calibration it was found that a movement of the writing lever of 0.30 cm. recorded a volume change of 1 cm.

Text-fig. 7 is a chart of this experiment and shows a smaller but characteristic result during the period of compression, due probably to the fact that in this case the lungs were free to expand within the thorax, having been collapsed away from the chest wall, whereas in former instances practically all space occupied by blood was alveolar.

Section 1, Fig. 4, shows the condition of the animal during the first normal collection indicated in Text-fig. 7. Section 2 of the same figure is the period of pulmonary vein compression. At the first upward mark on the base-line, tightening of the pulmonary vein clamp was begun. Between the second and third upward marks the air collection period *P* of Text-fig. 7 took place, and at the fourth upward mark the pulmonary vein compression was released. The cardiometer and blood pressure effects are as in former experiments. The tracing of intrathoracic volume rises promptly, indicating a swelling of the lungs with blood, and the individual excursions of the recorder become less owing to less extensive movements of the lungs with each respiration. The maximum rise is equivalent to an encroachment on the intrathoracic space of 6.6 cc. and the individual excursions are diminished by an amount equivalent to 1 cc. Section 4, Fig. 4, represents the conditions during the final normal collection period of Text-fig. 7.

Fig. 5 is a drawing of the posterior aspect of the lungs from this animal. The broken lines lead to darkened sections which indicate the extent of a slight area of congestion—an area not great enough, however, to cause any effect upon ventilation, since it is noticeable that the overflow collections in the last two periods of Text-fig. 7 are not greater than that obtained in the first period of the experiment.



Text-Fig. 7. Graphic representation of Experiment 4. Four 5 minute periods are shown.

DISCUSSION.

As indicated at the outset, these experiments were designed to determine whether pulmonary congestion interferes with the entrance of air into the lungs. Congestion was produced by compression of the pulmonary veins at their entrance into the left auricle, and the effect on the air entering the lungs was determined by means of a sensitive artificial respiration apparatus which delivered a constant volume of air with each inspiration. Any obstruction to the entrance of air into the lungs of the animal was indicated by a smaller volume of air being collected on expiration and a larger volume being collected in a spirometer connected with the inspiratory tube, arranged to receive the "overflow" which did not enter the lungs.

The experiments fall into two groups—those in which there was essentially no permanent change in the lungs as a result of the manipulations, and those in which extensive pulmonary congestion with edema into the air passages was produced. In the former, illustrated by Experiments 1, 2, and 4, constriction of the pulmonary veins and the consequent overfilling of the pulmonary circulation caused definite interference with the entrance of air into the lungs. In the different experiments the quantitative effect naturally varied within wide limits. That the effect on pulmonary ventilation was the result of vascular change alone is indicated by the fact that release of pressure on the veins resulted in a practically complete return to the previous normal conditions. The normal appearance of the lungs at the end of the experiments is also evidence that the whole effect was a vascular one, which corrected itself as soon as the obstruction to the circulation was removed.

In the second group of experiments, typified by Experiment 3, compression of the pulmonary veins produced not only the simple vascular effect which appeared in the first experiments, but also caused extensive pulmonary congestion with edema into the air passages. Marked permanent damage was done to the lungs. In these experiments constriction of the pulmonary veins produced an interference with the entrance of air into the lungs, as it did in the first group of experiments, but release of pressure was not followed by a return to normal conditions. The changes in the lungs resulting from the passage of fluid out of the vessels into the pulmonary tissues and the air spaces were not relieved when the pressure in the vessels was allowed to return to normal. In these experiments the blood pressure in the greater circulation fell progressively throughout the observation, and it seems probable that the more extensive and permanent damage to the lungs was in part due to the development of cardiac weakness. This was especially evident in Experiment 3, in which the extensive exudation was undoubtedly due to the combination of circulatory obstruction and weak heart muscle. The results obtained in this second group of experiments are in no way opposed to those obtained in the first group. On the contrary, they supplement them. The two sets of experiments show that if the pulmonary veins are obstructed to such a degree that congestion of the pulmonary vessels without exudation is produced, there results interference with the entrance of air into the lungs, which is relieved as soon as the obstruction is removed, but that if the circulatory conditions are such that exudation of fluid out of the vessels into tissues and air passages is produced, a permanent interference with the entrance of air into the lungs results.

There appear to be two ways in which pulmonary obstruction can act in order to encroach upon air space in the lung. First, the dilatation of the capillaries may actually take up alveolar space which air could occupy under normal alveolar conditions. This is a simple explanation of the observed change and readily accounts for the fact that less air entered the lungs on each inspiration. When the chest is closed and the lungs expanded so as to fill all the available space. it is clear that extra room which excess blood may occupy can only be obtained at the expense of the alveolar air space. The size of the lungs can only be changed, under these conditions, by pressures great enough to move the chest wall and diaphragm, and it is doubtful whether the right ventricle is capable of accomplishing such a result. When the chest is closed the lungs swell inwardly and large encroachment upon ventilation is the result. This change is illustrated by Experiments 1 and 2. When, however, the chest is open, blood may cause the lungs to swell outward, and under such circumstances the alveolar space may be very little changed and the amount of air entering the lungs not much altered. Experiment 4 illustrates just such a result and brings us to the second method by which pulmonary congestion may reduce the air space. It can be seen that in this experiment the expansibility of the lungs was diminished. The effect is, perhaps, as though the lung were erectile tissue rendered rigid and inelastic through vascular turgescence. Von Basch (3) suggested such an effect many years ago, and Experiment 4 is direct evidence of its existence but under abnormal conditions, the chest in this case being open. We are unable to say in what proportion these two factors operate to reduce ventilation in the closed chest, but are of the opinion that the first is the more important. It is possible that experiments now in progress, in which changes in capillary pressure are being measured, may throw light upon the situation.

We may now consider whether the facts derived from our experiments on animals afford any explanation of the conditions observed in patients with heart disease—conditions which were described at the beginning of this paper. In brief, it has been found clinically that patients with heart disease who become short of breath on exertion are unable to increase the depth of respiration, and that the vital capacity of the lungs is low. Experimentally it has been demonstrated that abnormal amounts of blood in the pulmonary circulation interfere with the entrance of air into the lungs. The clinical condition which has the closest analogy to our experiments is mitral stenosis. They are, indeed, essentially similar, for in mitral stenosis there is an obstruction to the flow of blood in leaving the left auricle, while in our experiments an obstruction was produced to entrance of blood into the left auricle. Both conditions, the clinical and the experimental, produce the same changes in the hemodynamics of the circulation in the lungs. In the early stages of mitral stenosis, when the obstruction to blood flow is slight, there is no abnormal tendency to dyspnea and no decrease in the vital capacity of the lungs. As the degree of stenosis increases, however, a tendency to dyspnea on exertion develops, and the vital capacity of the lungs is less than normal. Physical examination of the lungs reveals nothing to account for this interference with deep respiration. There is no exudation of fluid into the pleural cavities or into the air spaces, and thus no dullness on percussion, no alteration in breath sounds, and no râles. The conditions resemble those in Experiments 1, 2, and 4, in which there was engorgement of the pulmonary vessels with little or no exudation. The inability to breathe deeply and the low vital capacity, which is one of the outstanding features of mitral stenosis at this stage, may well be explained by engorgement of the pulmonary vessels and lung rigidity, just as these factors explained the interference with the entrance of air into the lungs in our experiments. At a later stage of mitral stenosis, physical examination reveals râles in the air passages and fluid in the pleural cavities. These indicate actual exudation of fluid out of the vessels, and the conditions are similar to those typified by Experiment 3, in which extensive areas of pulmonary congestion were found. In the clinical disease, as well as in the experiments, the development of actual exudation depends on two factors, the degree of obstruction to blood flow and the weakening of the heart muscle. At this period in clinical cases a still greater fall in vital capacity is found, and a greater tendency to dyspnea. The subject is unable to increase the depth of respiration enough to secure the degree of pulmonary ventilation necessary for aeration of the blood when the needs of the organism for oxygen are increased.

The relation of the conditions in the pulmonary circulation to the production of dyspnea is also suggested by other clinical observations. Mitral stenosis is the disease in which the most immediate effect on the pulmonary circulation would be expected, and dyspnea is an early symptom in mitral stenosis. Mitral insufficiency alone may not produce dyspnea for a long period of time, or apparently not until the heart muscle begins to weaken. The same is true of aortic insufficiency alone, but in association with a mitral lesion and involvement of the pulmonary circulation dyspnea begins to appear.

In aortic stenosis the situation is analogous to mitral stenosis, and when the pressure conditions are transmitted back to the left auricle and the pulmonary circulation, dyspnea becomes a prominent symptom. In all these diseases involving the heart valves the conditions are complicated by a coincident gradual degeneration or weakening of the heart muscle. No completely satisfactory analogy to this exists in our experiment for we were always dealing with a healthy myocardium. It was quite apparent, however, that the weakening of the heart in Experiment 3 acted in the same way as constriction of the pulmonary veins and served to increase the effects due to changes in the pulmonary circulation. It is not at all improbable that weakening of the myocardium alone may be enough to produce the same effects, and there is clinical evidence in harmony with this. Thus, in cases of myocardial weakness in later life the development of dyspnea on exertion is often the first symptom. This is usually accompanied by a fall in vital capacity, but at first there are no physical changes of importance in the lungs. Later, râles and pleural effusion appear. Dyspnea commonly develops early in such cases, while edema of the legs and hepatic enlargement come later. This suggests that in many cases of myocardial weakness the failure of the circulation takes place first in the pulmonary circuit. Clinical experience, as well as the experiments which have been described, indicates that in the first stages there may be interference with the entrance of air into the lungs without the production of physical signs of lung involvement. At a later stage pulmonary edema and pleural effusion will take place.

SUMMARY.

1. A method is described for producing pulmonary congestion, together with what may be termed a differential spirometer method for studying lung ventilation.
2. The method utilized permits an approximately accurate prediction of degrees of pulmonary edema in the living animal, and suggests avenues of approach for the very difficult problems of pulmonary capillary pressure.
3. It is shown that intravascular blood can encroach markedly upon the pulmonary air space. Although the methods used in these animal experiments do not resemble vital capacity measurements in man, their result is so definite that their applicability to clinical conditions may be considered.
4. The similarity between the experiments described and certain conditions of cardiac decompensation, of which mitral stenosis is the best example, is pointed out.

BIBLIOGRAPHY.

1. Siebeck, R., *Deutsch. Arch. klin. Med.*, 1910, c, 204.
2. Peabody, F. W., *Am. J. Med. Sc.*, 1918, clv, 100.
3. von Basch, S., *Verhandl. deutsch. Cong. inn. Med.*, 1889, viii, 384.
4. Drinker, C. K., *J. Exp. Med.*, 1921, xxxiii, 675.
5. Schafer, E. S., *Quart. J. Exp. Physiol.*, 1918–20, xii, 133.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Kymographic record covering collection periods 2, 4, and 5 of Experiment 1. Upper line of tracing, marked 1, is the cardiometer record; tracing 2, the rate of artificial respiration; tracing 3, arterial pressure, mercury manometer, femoral artery; tracing 4, the base-line for blood pressure. Upward marks on this line signal events referred to in text; downward marks, 15 second intervals—inaccurate in this instance owing to defective clock. The tracing is reduced to about $\frac{1}{4}$ (it is actually $\frac{44}{175}$) of the original size.

FIG. 2. Kymographic record covering collection periods 1, 3, and 4 of Experiment 2. Lines of tracing similar to those in Fig. 1. The tracing is reduced to about $\frac{1}{4}$ (it is actually $\frac{44}{175}$) of the original size.

PLATE 2.

FIG. 3. Kymographic record covering collection periods 4, 5, 6, and 7 of Experiment 3. Lines of tracing similar to those in Figs. 1 and 2. The tracing is reduced to $\frac{44}{175}$ of the original size.

FIG. 4. Kymographic record covering collection periods 1, 2, and 4 of Experiment 4. Lines of tracing similar to those in Figs. 1 to 3, except that line 2 records intrathoracic volume changes and time marker records 5 second intervals. The tracing is reduced to about $\frac{1}{4}$ (actually it is $\frac{44}{175}$) of the original size.

PLATE 3.

FIG. 5. The lungs of the animal used in Experiment 4. Dotted lines lead to area of congestion described in text.

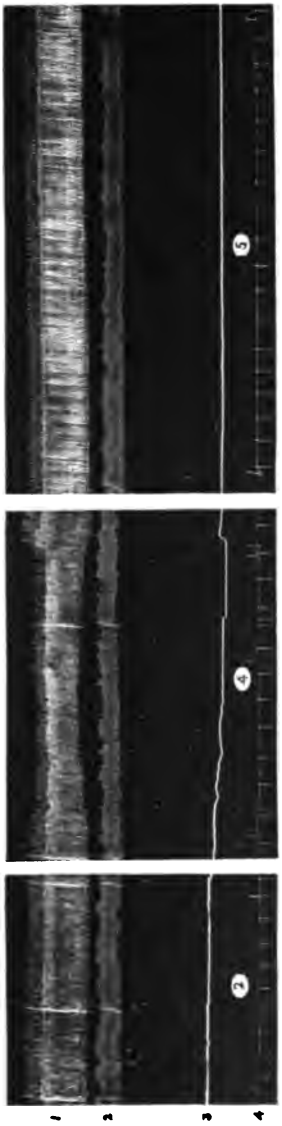


FIG. 1.

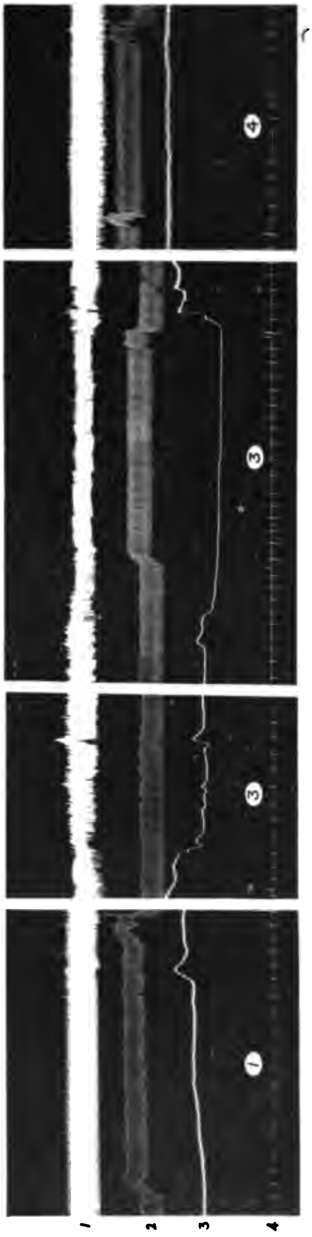


FIG. 2.

(Drinker, Peabody, and Blumgart: Pulmonary congestion.)

EXPERIMENTAL GENERALIZED ANALGESIA AFTER EXPOSURE TO SOME WAR GASES.*

BY JOHN AUER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 4 TO 6.

(Received for publication, June 28, 1921.)

During a series of investigations, taken up at the instance of the Surgeon General of the Army, concerning the functional effects following exposure to dimethylsulfate and chloropicrin in a gaseous or finely subdivided state, it was noted accidentally that these two war gases reduced pain perception to an astonishingly low level. This analgesia was so profound in many cases that a laparotomy could be performed without any indication of pain and without markedly influencing the blood pressure. As this effect seems to be practically unknown, numerous experiments were carried out to test pain perception in gassed cats. The results justify the statement that the two war gases mentioned strikingly reduce pain perception in cats under the conditions which will be described.

The only reference in the literature of war gases bearing on this aspect which I have been able to find, is an article by Naiding.[1] This author had the opportunity of examining soldiers gassed with chlorine and phosgene, and states, among other observations, that numbers of them exhibited a general or localized loss of pain perception; thus, he noted that the analgesia involved the skin of the whole body in some, while in others it was localized in the forehead, scalp, hands, or feet. There is no record that deep pain perception was tested.

---

* A preliminary note was published in the *Proceedings of the Society for Experimental Biology and Medicine* (Auer, J., *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 104). The final publication was delayed by conditions beyond the control of the author.

This work forms part of some war gas investigations which began in 1917 and were undertaken at the suggestion of Dr. Simon Flexner and Dr. Henry D. Dakin. I am under special obligation to Dr. Dakin for numerous helpful suggestions concerning methods of gassing, precautions to be observed, and also for kindly supplying me with chloropicrin.

[1] Naiding, M. N., *Russk. Vrach*, 1917, xvi, 391.

Methods.

Cats only were utilized in this investigation for reasons of convenience. The gas chamber was of the ice box type with a cubic capacity of 200 liters. In two of the walls large glass windows were sealed so that the entire chamber could be readily inspected at any time; fogging of the glass panes was prevented by using a commercial soap sold for this purpose. One side of the chamber was formed by a completely removable door. Gas tightness was obtained by carefully filling all visible cracks in the wall of the inner chamber with putty and then applying several coats of white enamel. The door was made gas-tight by providing two soft rubber gaskets, one glued on the door and the other to the box. Before closure the contact surfaces were covered with vaseline. Complete contact was assured by four lever handles on the box which fitted in corresponding hooks on the door.

The cats were gassed in a wire cage, this container being placed on brackets in the chamber. The brackets were about 5 inches from the floor of the chamber so that the animal was at the same level as the window during the gassing period. Under the wire cage an enameled pan was placed to prevent soiling of the chamber.

The heat necessary for vaporizing the dimethylsulfate or chloropicrin was furnished by an electric hot-plate which could be set to reach 250°C. This plate was located in one corner on the floor and its switch was placed on the outside of the chamber. The hot-plate also served as a convenient means of maintaining the chamber temperature between 25° and 30°C. The temperature was read from a thermometer suspended near a window in the chamber.

As the gases employed were heavier than air, a small motor-driven fan was screwed in a corner of the chamber. This fan was kept in motion constantly throughout the experiment and prevented the gas vapors from settling to the floor.

The duration of gassing was kept constant for each of the various series of experiments. With dimethylsulfate the cats were gassed for 1 hour in the great majority of experiments. With chloropicrin the duration of gassing was either ½ or 1 hour in most cases.

The concentration of gas chosen was such that death resulted in a majority of the animals within 1 to 4 days after gassing. This was determined by a series of trials and was as follows for the two gases: for dimethylsulfate 4.5 to 10,000 air; for chloropicrin 1 to 10,000 air.

The amount of liquid necessary to produce the required concentrations was readily calculated. Thus 20 cc. of gas were needed to give 1 to 10,000 concentration in a chamber of 200 liters capacity. As the molecular weight in grams of each liquid produces roughly 22 liters of gas, the weight of liquid required for 20 cc. of gas was determined and then translated into measure by volume. Curiously enough, the volumes of dimethylsulfate and chloropicrin necessary to produce

20 cc. of gas were identical: 0.087 cc. for dimethylsulfate and 0.088 cc. for chloropicrin. For practical purposes 0.09 cc. was considered the amount necessary for a 1 to 10,000 concentration. No corrections were made for temperature.

From these conditions it will be seen that the calculated concentration of the war gas would only be maintained for a short time, because condensation and droplet formation would begin shortly after vaporization. The gas concentration would then necessarily sink. This was not considered a drawback because the same conditions were maintained as far as possible throughout all the experiments; moreover, it is believed that this arrangement presents a closer approximation to conditions existing on the battle-field after the explosion of gas shells, than the ordinary procedure in which the desired gas concentration is drawn through the testing chamber.

Dimethylsulfate, $(CH_3)_2SO_4$, is a liquid at ordinary temperatures with a specific gravity of 1.32 and a boiling point of 188°C. Chloropicrin, or trichlornitromethane (CCl_3NO_2), is also a liquid and possesses a specific gravity of 1.69 and a boiling point of 112.8°C.

The chamber was evacuated by suction after the gassing period, the door of the chamber being perforated by two stop-cocks with ¼ inch lumen. These cocks were located in the upper and lower parts of the door respectively. The chamber gases were drawn off through the lower cock, while the upper one was open. Suction was furnished by the house vacuum system. Before the war gases were allowed to enter the vacuum system, they were passed through absorbents. For dimethylsulfate vapors 30 per cent NaOH wash bottles and soda lime tubes were used; for the chloropicrin gas, charcoal tubes were introduced into the system. The routine time allowed for evacuation was 30 minutes.

Thick rubber gloves were worn while removing the cage from the gas chamber and while cleaning it and the wire cage. The laboratory helpers were also encouraged to wear the regulation army gas mask (older form with nose clip) as a routine measure during the process of gassing; an hour's trial, however, invariably brought the conviction that this additional factor of safety was superfluous.

The precautions observed may seem excessive when it is considered that fractions of a cubic centimeter of the war liquids sufficed to give the desired gas concentration in the chamber, and that this amount completely vaporized in an average sized, well ventilated room would be entirely devoid of danger. The precautions, however, were made obligatory to counteract the depressing mental effect which war gas work exerted initially upon the helpers.

The methods employed in testing the analgesia produced by dimethylsulfate and by chloropicrin will be described in the section dealing with the experiments.

General Picture.

Before entering upon the subject of this paper it is necessary to give a brief description of the chief reactions which the cat exhibits during and after the period of gassing. While being gassed with dimethylsulfate in a concentration of 3 to

5 per 10,000 of air, the cats show a swiftly increasing irritation of the respiratory mucous membranes and of the conjunctiva. There is, first, winking of the eyelids, narrowing of the lid aperture, licking of the nose, and increased tear secretion. A little later the eyes are kept more or less closed. Sneezes and slight coughs now are noticed and a clear, watery saliva drips from the jaws. This watery saliva is soon replaced by a viscous secretion which hangs in gelatinous ropes from the angles of the mouth. At this stage the mouth is kept open most of the time, as apparently the nasal passages are obstructed. The respiration of the cat varies from 20 to 27 per minute and is not labored; towards the end of the gassing period most animals show short spells of polypnea, during which the tongue is protruded and the cat pants like a dog. Vomiting may occur during the last half of the gassing period or shortly after removal from the gas chamber. In general there is only a moderate restlessness at the start and then the animal lies or squats in a normal position on the floor of the cage. Certain animals, however, exhibit considerable excitement while being gassed, and this was more frequent among females than among males. The excitable cats were generally more severely affected by the same concentration than their more stolid mates. The mucous membranes of the mouth, and the tongue were usually red-pink throughout the gassing period, only occasionally was a slight cyanosis observable. It is worthy of note that most of the cats seemed astonishingly comfortable on the whole in the gas chamber.

After removal from the gas chamber the cats appeared to be in excellent general condition, but were disinclined to make any unnecessary movement; some muscular impairment was shown occasionally by a slight stagger on walking and by the short duration and weakness of resistance when examined. The animals preferred to crouch quietly in one place with head slightly extended and eyes closed. Inspection showed the jaws slightly open; slight or no salivation; moderately slow respiration, about 25 or 30 per minute, accompanied by rhonchi; expiration was slightly prolonged and active. On gently separating the lids the corneas generally exhibited a centrally located, oval, milky opacity, and the conjunctivæ and nictitating membranes were reddened and swollen. Auscultation revealed the lungs filled with snoring, groaning, clucking râles. These râles largely obscured the heart sounds. The blood pressure in the femoral artery was always at least 100 mm. Hg., generally more. From now on the course was usually progressively downward. The respiration became more and more labored; inspiration was slow and accompanied by a wide opening of the jaws; occasionally some retraction of the costal margins was observable during inspiration; expiration was usually prolonged and active. The mucous membranes were usually of a good color, but slight exertion of the animal sufficed to bring on a definite cyanosis. Death generally took place within 4 days, though some cats survived for months.

No food or water was taken by the cats which succumbed in 3 to 4 days; any attempt to swallow apparently led to choking.

Autopsy revealed a marked inflammation of the entire respiratory tract. The pharynx was reddened and occasionally covered with patches of yellow exudate;

the epiglottis was reddened, edematous, and occasionally covered with a gelatinous exudate. In some instances the tip of the epiglottis was occupied by a vesicle of edematous tissue. The entrance to the larynx may be a mere slit in a waterlogged mass of tissue.

On opening the trachea and bronchi the entire mucosa was found covered by a grayish, gelatinous, thick membrane which extended apparently into the finer bronchioles. This membrane could be readily removed and formed a tough mass; frequently it was possible to obtain what seemed to be perfect tubular casts of a large part of the bronchial tree. The thickness of this pseudodiphtheritic membrane was often so great that the tracheal lumen on inspection seemed largely occluded, and some of the smaller bronchi were apparently completely plugged. Pulmonary edema was not frequent, and when present was remarkably localized, involving only one lobe or only a portion of a lobe.[2]

Chloropicrin gas in a concentration of 1 to 10,000 for 30 minutes or 1 hour causes symptoms which are in general similar to those obtained with dimethylsulfate. The main differences will appear in the following brief description.

As soon as the cat or cats are introduced into the gas chamber and before the chloropicrin has been heated, the animals wink rapidly and salivation begins. Within 1 minute ropy masses of saliva hang from the angles of the jaws; oftentimes clear, watery saliva drips over these glairy appendages. The salivation reaches a maximum and towards the end of the gassing period may have largely disappeared, as in dimethylsulfate intoxication. Retching and vomiting may occur early and is more frequent than with dimethylsulfate. During the early period of gassing a moderate restlessness is apparent. This is followed by a stage of quiescence during which the cat squats or crouches in the cage. Occasionally a short fit of restlessness intervenes. The respiration at the beginning of gassing is about 25 per minute; towards the end of the period it is generally shallower and faster, 50 per minute, with a slight active expiration. The prolonged active expiration seen with dimethylsulfate was absent, nor was opening of the jaws with inspiration such a prominent feature during and after chloropicrin gassing as when dimethylsulfate was employed. There was no evidence of marked asphyxia throughout the experiment, judging from the pink color of the tongue and mucous membranes of the mouth.

After removal from the gas chamber the cats were quiet and only slightly salivated. The respiration often was composed of short, weak inspirations followed by a sudden, sharp, active expiration. Auscultation showed the chest filled with coarse râles, which obscured the heart sounds. Muscular weakness was shown by feeble resistance to extension and by a disinclination to move. In the majority of instances death occurred within 48 hours after gassing with chloropicrin 1 : 10,000 for ½ to 1 hour; a few cats, however, survived for months.

The autopsy exhibited a picture quite different from that produced by dimethylsulfate. The larynx and trachea were moist and pale, without any congestion or membrane formation. Foam and fluid were generally found in the trachea; the lungs were large, heavy, and mottled with moist brownish areas and fresh hemorrhages. On closer examination these brown areas were seen to be areas of lung alveoli completely filled with fluid; the borders of the lobes were often emphysematous. On section, fluid poured from the cut surface as if from a saturated sponge. The parenchyma also often revealed numerous and large hemorrhages. On the surface of the section each bronchus and bronchiole was surrounded by a translucent, moist, gray zone of edema and the lymphatic trunks near the hilus were always engorged with clear fluid. This engorgement of the pulmonary lymphatics is observable at a time when the lung alveoli contain but traces of edema fluid, for example in cats killed after 15 minutes exposure to the gas. The edema was usually most pronounced in the lower lobes, though all lobes were involved. The degree of edema was often so great that moderate compression of the chest caused a gush of liquid from the trachea.

---

[2] Auer, J., *Proc. Soc. Exp. Biol. and Med.*, 1917-18, xv, 106.

The diaphragm level was often abnormally low, due to a tonic contraction of its muscle fibers as indicated by the ridge formation of the muscle tissue at its tendinous insertion. This tonic contraction was also observed in cats in which a biopsy was carried out, and is perhaps ascribable to an *intra vitam*, partial rigor.

The heart muscle was usually brownish in color and flabby. On section, macroscopic hemorrhages in the left branches of the His bundle were frequently observed.

Analgesia.

Reduction of the capacity to perceive ordinarily painful stimuli was first observed during a tracheotomy performed to save an animal from the effects of a laryngeal stenosis. This animal had been gassed with dimethylsulfate 24 to 36 hours previously when suddenly it showed restlessness, the tongue and mucous membranes became bluish, the respiration was labored, and with each inspiration the costal margins now sank inwards. Tracheotomy was carried out at once and the symptoms were relieved.

Before describing the tests it is important to emphasize that all the manipulations were carried out with the greatest care and were always executed in a fixed way. The mild tests were invariably applied first and if they indicated analgesia, other procedures were cautiously attempted. As soon as a definite restlessness developed the test was discontinued. The cats were usually killed by injecting 10 to 15 cc. of ether into the trachea.

The tests were carried out while the cats were either lying or squatting on a table or when they were extended back down. A sharp, brief pressure of the pinna of the ear, the nasal septum, lips, the skin of the body, the toe pads, and tail by means of a hemostat caused in typical cases no sign of pain. After the stimulus, the compressed ear was flattened against the skull, the head drawn to one side, the foot pulled away, or the tail twitched aside. In the normal cat a sudden moderate pressure upon the tail usually calls forth a short, strident yowl, an arching of the back, and a sidewise retreat while the animal spits a few times and glares with apprehensive resentfulness at the experimenter.

With the greatest precaution further tests were then made. It was found that the carotid or femoral artery could be exposed and freed from the accompanying nerves, that large flaps of skin could be resected, and that the tibialis anticus muscle and tendon could be dissected free, without causing any or only slight and indefinite evidences of pain. These evidences consisted chiefly of a slight restlessness and probably denoted discomfort rather than pain, for normal cats are not diffident about responding vocally to traumatic stimuli.

Still further experimentation showed that the peritoneal cavity could be opened in the midline; that, furthermore, a loop or loops of intestine could be delivered through the laparotomy wound and gently pulled, or that the parietal peritoneum could be rubbed with the finger without eliciting more than a slight general rolling motion of the body (Figs. 2 and 3).

However, marked stretching of a mixed nerve or of the mesentery, a strong electrical stimulation of an afferent nerve, all produced signs which could be interpreted as evidences of some perception of discomfort or even pain: the animal became moderately restless, the respiration quickened and deepened, and the blood pressure exhibited some elevation of its level.

The analgesia was generally well established within a few hours after gassing and within 24 hours reached a maximal degree. These animals were often in an apparently excellent condition, and Figs. 1 and 2 present an autographic record of how animals of this type fail to respond to ordinarily painful stimuli.

The record of Fig. 1 was furnished by a cat which had been gassed with dimethylsulfate in a concentration of 4.5 to 10,000 air for 1 hour. This animal was a male, orginally weighing 4,150 gm., and had been fed shortly before gassing. 26 hours after gassing he was in good general condition, though respiration was slow, slightly audible, and accompanied by some retraction of the costal margins; expiration was active and prolonged; the respiration rate was 32 per minute; his voice was hoarse. There was no obvious muscular weakness and the animal jumped with ease and coordination from the examining table to the floor. No food had been taken since exposure to the war gas, and his weight now was 3,760 gm., showing a loss of 390 gm. As soon as the animal was extended upon his back, the inspiration became more labored and snoring, and retraction of the costal margins increased in extent. The trachea was now exposed and partially slit transversely; the dyspnea was at once relieved, the inspiration now was unlabored and no longer accompanied by a snoring sound or by a costal retraction; expiration was also easier but was still moderately active. The trachea was surrounded by a zone of emphysema and the tracheal lumen was partly obstructed by a thick, gelatinous, membranous sheath, a part of which was removed.[3] The operation developed not the faintest sign of pain; the animal was perfectly quiet and the tongue and mucous membranes had a better pink color than before. The left tibialis anticus muscle and left peroneal nerve were now completely dissected free and the nerve cut without any sign of pain; the cat rested quietly on its back and only the respiration showed a transitory acceleration in rate after section of the nerve. The animal was then prepared for recording the blood pressure in the right femoral artery. The section of the skin, the freeing of the artery from the crural nerve by blunt dissection, the placement of the nerve on a loop of thread, all caused no restlessness and the animal remained as quiet as if under the influence of an anesthetic. Stimulation of the intact crural nerve with the faradic current (300 mm. coil distance, two Daniell cells, Petzold coil, cable electrodes) induced a slight rolling motion of the animal accompanied by a blood pressure rise of 13 mm. Hg. (Fig. 1). Slight movements of the cat also caused elevations of the pressure by about 20 mm. Hg.

---

[3] Removal of the tracheal membranous exudate is always dangerous because a section may be aspirated and plug the trachea and bronchi. For the same reason a transverse section of the trachea should never completely sever the tube of membranous exudate.

Exposure and severance of the left vagosympathetic nerve were painless. The cardiac vagus endings exhibited perhaps some reduction of sensitiveness, for a coil distance of 100 mm. was necessary to obtain the characteristic slowing of the heart (Fig. 1). The motor vagus endings in the esophagus reacted vigorously when the peripheral vagus was electrically stimulated with the coils 150 mm. apart. Stimulation of the central vagosympathetic trunk produced characteristic respiratory effects: slowing and deepening of the respiration associated with inspiratory stoppage broken by short vigorous expirations when a strong current was used (150 mm. coil distance; see Fig. 1). No respiratory stoppage in expiration was observed with the current strengths employed. The blood pressure exhibited the characteristic depressor type of fall when the left central vagosympathetic trunk was stimulated. The depressor portion of the vagosympathetic trunk was exceedingly sensitive, mere placement of the nerve stump on the platinum electrodes sufficed to cause a fall of 20 mm. Hg. (Fig. 1). The eye responded well to stimulation of the left central vagosympathetic trunk; with 250 mm. coil distance the nictitating membrane retracted slowly, but the pupil showed no dilatation; with 200 mm. the nictitating membrane retracted well and the pupil dilated strongly; on stoppage of the stimulation, the pupil contracted promptly to its original slit-like form. The tibialis anticus muscle exhibited no sign of fatigue when stimulated indirectly (peroneal nerve) with short tetanizing currents, 150 mm. coil distance, at 2 second intervals for 6 minutes. After 1 hour the rectal temperature was 35.7°C.

The depth of the analgesia was still further tested by opening the peritoneal cavity in the median line. There was no evidence of pain and the cat remained practically immobile. Insertion of the finger into the peritoneal cavity and gentle friction of the parietal peritoneum caused no resistance. The arteries of the intestine were bright red, while the veins were quite dark in color. The animal was then killed.

Quite similar results were obtained when chloropicrin was employed. The following abstract of a typical protocol, together with Fig. 2 giving the blood pressure record, will demonstrate this fact.

This cat was a non-pregnant female weighing 3,750 gm. and had been fed previously; chloropicrin in a concentration of 1:10,000 was employed for 30 minutes. 23 hours afterwards the cat weighed 3,180 gm. but was in apparently good condition, though the respiration was moderately labored, with slightly snoring inspiration, about 44 to the minute, and was accompanied by an expiratory grunt. The expiratory grunt only became audible some time after expiration had begun. The tongue and mucous membranes were slightly cyanotic. The ordinary pressure tests yielded the same result which has been described in the dimethylsulfate animal. In this animal the dorsal position also increased the respiratory difficulty: the tip of the sternum now exhibited a definite depression with each inspiration and a moderate restlessness appeared. Tracheotomy was carried out at once and a fair amount of brownish fluid and foam escaped from the tracheal wound, which was increased by moderate compression of the chest and elevation of the foot of the board. As soon as the trachea had been slit the restlessness of the animal ceased, and it remained quiet; the tongue and mucous membranes became red without any trace of cyanosis. Some respiratory stenosis still existed, however, for the sternum still showed a slight depression with each inspiration. The right femoral artery and the left vagosympathetic trunk were also exposed. Fig. 2 shows the blood pressure record of this animal before, during, and after stimulation of the left peripheral and central vagosympathetic stumps; also before and after opening the peritoneal cavity and while gently rubbing the parietal peritoneum. The blood pressure curve of this animal reveals no significant changes while the parietal peritoneum was rubbed for approximately 10 seconds by the finger. The cat did not even move, for every movement is betrayed by a rise of pressure. The response of the cardiac vagus to electrical stimulation occurred with a weaker current in this cat than in the preceding animal; 200 mm. coil distance sufficed, while 100 mm. were necessary in the previous cat before a cardiac slowing was noticeable. The reflex respiratory effects in the chloropicrin cat after stimulation of the central vagus showed active expiratory stoppages and not inspiratory stoppages as in the dimethylsulfate animal. During these respiratory stoppages in active expiration foam and fluid were occasionally expelled from the trachea. The depressor nerve was not as sensitive in this animal as in the dimethylsulfate cat. On the other hand, the chloropicrin cat exhibited a much greater sensitiveness of the dilator fibers of the cervical sympathetic, for a coil distance of 400 mm. sufficed to give a definite pupillary dilatation, while 250 mm. was the weakest current which was effective in the dimethylsulfate cat.

In this connection it must be mentioned that this cat exhibited definite hippus before tracheotomy had been carried out; the pupils dilated during inspiration and contracted well during expiration. The pupils responded to light and varied in diameter as the animal looked around (accommodation). The lid reflex was prompt. The arteries of the intestine were red, while the veins were moderately dark. The animal was killed later.

Autopsy of this cat showed a strong pulmonary edema; the lower lobes were full of fluid while the upper lobes contained less fluid but more foam and air.

These two abstracts and tracings (Figs. 1 and 2) reveal unmistakably that the capacity for pain perception, both superficial and deep, may be enormously reduced by exposure to the two war gases mentioned. The degree of analgesia exhibited by the two cats described above was not observed in all animals but in the great majority the presence of a well defined, undoubted analgesia could be readily established.

The duration of the analgesia could not be determined with accuracy because most of the cats succumbed within 4 days. After chloropicrin intoxication a number of cats exhibited, about 5 days after gassing, outspoken signs of pain perception when mild pressure tests were applied to the tail. The reaction was so definite that no further tests were made. The same reestablishment of pain perception was also noted in a chloropicrin cat which survived for 5 months. After dimethylsulfate, on the other hand, the analgesia may still be present to a remarkable degree even after the lapse of 6 months. Fig. 3 illustrates the one instance I have observed.

This cat was a male weighing 4,250 gm. originally, and had been subjected to the effects of 4 parts of dimethylsulfate to 10,000 of air for 1 hour. The effects were severe and the animal did not show a change for the better until 7 days after gassing. 10 days after gassing, food was taken more regularly and the animal moved about in a practically normal fashion. 6 months after gassing, the animal was still alive though markedly emaciated, and weighed 2,890 gm., which represents a loss of 1,060 gm. The emaciation was not due to failure to eat, for the animal ate heartily. There was no definite muscular weakness discernible and he walked and jumped on occasion like a normal animal. The nose showed a yellowish fluid discharge. Both corneas were spotted with a central staphylomatous leucoma and synechias stretched between the upper pole of the iris and the cornea of each eye. The respiration was slow and easy, with slightly prolonged expiration, and the rate was 40 per minute. Moists throaty sounds often accompanied the respiration. The usual test, were carried out and no definite signs of pain perception were elicited. Fig. 3 represents the blood pressure curve of the animal while being tested for deep pain perception. Both vagosympathetic trunks were intact and no tracheotomy was performed in this cat. It will be observed that a pull on the crural nerve caused only a slight short rise of pressure which was considerably less than that obtained when the animal was slightly restless just preceding this test. Opening of the abdominal cavity caused no change in the pressure level worth mentioning nor did restlessness occur. That the procedure had caused reflexly effective afferent impulses is seen, however, in the rise of the pulse rate from 140 to 160 and in the increase of the respiratory rate (25 to 32 per minute). Introduction of the finger into the abdomen caused a short rise of the blood pressure from 126 to 148 mm. Hg. and was associated with slight general movements. These reactions surely indicate that sensory impulses reached the higher centers, but from the general behavior of the cat it seems equally certain that these sensory impulses were perceived as uncomfortable sensations rather than as pain sensations. The cat was then killed by means of ether.

DISCUSSION.

The observations reported on preceding pages indicate clearly that cats gassed with chloropicrin or dimethylsulfate under the conditions mentioned do not react normally when subjected to stimuli which are painful under ordinary conditions; operative interferences may be undertaken, involving the skin, the deeper interfascial structures of the leg and neck, or the peritoneum even may be incised and its parietal leaf rubbed, without eliciting the ordinary signs of pain. In other words, gassing cats with one of these two war liquids endows the treated animals with a marked degree of analgesia both for superficial as well as for deep pain stimuli.

The question now arises how this experimental analgesia is produced, whether there is any single factor at work which may explain this curious effect of at least two, chemically different war gases. There are four possibilities to be considered: (1) anesthesia of the sensory nerve endings of the skin; (2) blocking of sensory impulses in the nerve trunks; (3) that the analgesia does not exist in reality but is simulated by the fact that motor weakess prevents expression of the normal reaction to pain stimuli; and (4) that the higher centers are largely unable to perceive pain stimuli. It must be stated at once that some of these factors can be excluded with considerable certainty, yet perhaps all may be involved to some degree in the causation of the final result; on the whole, however, one factor appears to play a dominant rôle. The various possibilities will now be discussed briefly.

It might be assumed that the receptive sensory endings in the skin are damaged by the war gas vapors which condense to some extent at least in the fur of the animals. Such a condensation apparently does take place, for the behavior of fleas, with which most of the cats were infested, indicates this. Shortly after gassing the fleas always became restless, left the deeper portions of the fur, and climbed to the extreme tip of the longer hairs where they maintained a precarious position with difficulty until increasing weakness caused them to fall or jump feebly to the floor of the gas chamber where they died. The experimental evidence at my disposal does not permit any statement as to whether or not the two war gases produced the postulated skin anesthesia, but the facts already submitted show definitely that such an anesthesia fails to explain the presence of analgesia to deep pain stimuli (stimulation of nerve trunks, opening of the peritoneal cavity; see Figs 1 to 3). The localized analgesia observed in gassed soldiers by Naiding would seem to indicate, however, that under certain conditions some war gases may destroy the functional activity of the sensory skin endings. It is possible that this local action was reinforced by a systemic effect, for Naiding states that

80 per cent of his patients complained of pain in the chest, asthma, or coughing.

The second possibility was a blocking of sensory impulses in the nerve trunks subsequent to the inhalation of the war gases. The experiments and tracings give no support to this supposition. In numerous instances typical analgesia was present when examination revealed a prompt knee jerk, a pupil responding normally to light, accommodation and sympathetic nerve stimulation, and prompt, effective, medullary reflexes when the central vagus and depressor nerves were stimulated (see Figs. 1 and 2). These results would be impossible if conductivity in the nerve trunks were strongly reduced. Whether or not, however, a qualitative blocking for pain stimuli existed cannot be discussed profitably in the present state of our knowledge.

The third possibility considered the analgesia as non-existent in reality and assumed that peripheral motor weakness prevented the normal response to pain stimuli. This explanation is absolutely disproved by the fact that stimulation of a motor nerve produces normal muscular contractions, and by the further fact that the gassed muscle, for example the tibialis anticus, may show little if any greater fatigability than a normal muscle when stimulated directly, or indirectly through the severed peroneal nerve, the muscle being left *in situ* with blood and motor nerve supply intact.

There remains for consideration the fourth possibility, that the higher centers are largely unable to perceive pain stimuli as such, and this assumption satisfactorily explains all the observed facts. The foundation for this belief has already been presented by exclusion in the discussion of the three explanations of war gas analgesia, all of which placed the cause in the periphery. The dismissal of these peripheral causes as inadequate or inadmissible compels the view that the cause must be located in the higher centers. The higher cerebral centers, therefore, after gassing with dimethylsulfate or chloropicrin are apparently so altered that ordinary pain stimuli are no longer recognized as such, and this alteration does not appreciably interfere with execution of most of the basic reflexes which link the functional organism into a unit.

What the nature of this alteration may be can be inferred to some extent after determination of the active agent which causes these specific changes in the higher nerve cells. In the search for this agent it might be assumed that the war gases producing analgesia perhaps show similar chemical groupings or structures and that this is causally related to the production of analgesia. There is no definite basis for this supposition beyond the fact that the three gases which have been described as producing analgesia, dimethylsulfate ($(CH_3)_2 SO_4$), chloropicrin ($CCl_3 NO_2$), and phosgene ($COCl_2$), may all be considered methane derivatives. The observation, however, that generalized analgesia may persist for 6 months after gassing (see Fig. 3) renders the view quite improbable that the war gas itself is the agent directly producing the analgesia. As far as I know there is no volatile chemical substance, excluding sublimation of the heavy metals, which can exert an effect in the body lasting for months after a single administration. It is therefore necessary to seek among the secondary effects of war gases for the common agent which produces analgesia. This common agent I believe to be a general, persistent, low grade asphyxia. The reasons are as follows:

It was noted that moderate exertion of short duration changed the pinkish buccal mucous membranes of the resting cat to a bluish cyanotic color; a mere change of position, for example extension on the back, often sufficed to bring on a notable cyanosis associated with restlessness, dilatation of the pupil and increased dyspnea; all of these undesirable results generally could be relieved by tracheotomy. Autopsy of these animals revealed the pulmonary air passages largely filled with a membranous exudate or with edema fluid. It therefore seemed clear that during life the lung ventilation barely sufficed for the vital needs of the organism, and that a small additional respiratory difficulty or increased production of CO_2 (struggles) brought on an obvious asphyxia. Furthermore, there seemed to be a definite association between the presence of marked analgesia and the functional capacity of the lung. Thus a number of chloropicrin survivors exhibited normal reactions to moderate pressure stimuli about 6 days after gassing. These responses were so clear that further tests were not deemed justifiable and were therefore omitted. After killing these cats with chloroform, examination showed the lungs to be in good functional condition; the color was pink, they collapsed normally on opening the chest cavity, section revealed no trace of edema, and there was no stenosis of the air passages. The only marked abnormality was atelectasis of one or more of the upper lobes. In these cats, therefore, pulmonary ventilation of the blood was practically normal and no asphyxia existed. On the other hand, a dimethylsulfate survivor exhibited a typical analgesia 6 months after the date of gassing (see Fig. 3). Autopsy of this cat exposed a voluminous lung which collapsed but slightly on opening the chest; its borders were emphysematous and section disclosed the pulmonary passages including the trachea, apparently completely filled with a yellowish, semifluid, gelatinous exudate resting on a tough membranous base. The mechanical obstruction in the trachea, bronchi, and bronchioles was such that pulmonic ventilation seemed impossible, and yet no signs of respiratory distress were observed while the cat was walking about; indeed the cat seemed quite comfortable for it often raised its tail to a vertical position, rubbed itself against the observer, and made moist, purring sounds. In this cat a low grade, chronic asphyxia surely existed.

The various facts presented above legitimize the inference that a systemic, low grade asphyxia of pulmonic origin is a primary factor in creating and maintaining the generalized analgesia described in this paper. Other factors probably also contribute to the final result. Among these, inspissation of the blood[4] due to edema, salivation, vomiting, and deficient fluid intake, and cellular changes[5] in various parenchymatous organs of primary or secondary origin, may be of some value. The formation of methemoglobin apparently does not take place to any extent with the doses of dimethylsulfate and chloropicrin employed in these experiments. The most asphyctic blood readily turned to a bright red color when lightly shaken with some Ringer solution; moreover, Gildemeister and Heubner[6] report only doubtful spectroscopic signs of methemoglobin in the blood of cats, even when the doses of chloropicrin were so large that death resulted within 15 to 18 minutes.

---

[4] A careful study of various blood changes after chloropicrin, phosgene, and chlorine inhalations in the dog has been made by Underhill (Underhill, F. P., The lethal war gases: physiology and experimental treatment, New Haven, 1920).

[5] See Gildemeister, M., and Heubner, W., *Z. ges. exp. Med.*, 1921, xiii, 325, for a description of cellular changes after chloropicrin.

[6] Gildemeister, M., and Heubner, W., *Z. ges. exp. Med.*, 1921, xiii, 315.

The analgesia described in the preceding pages may be of some practical importance in the human subject. In gassed soldiers, for example, where surgical intervention is necessary, it is quite probable that little or perhaps even no general anesthetic may be necessary. This would be desirable because it removes, for some patients at least, the danger of a general anesthesia by means of ether or chloroform in a partially asphyctic individual. Another benefit would be a conservation of the immediately available supply of anesthetics, a factor of some importance for stations in the field of operations where transport facilities are never ideal.

SUMMARY.

Cats gassed with dimethylsulfate or chloropicrin in such concentration that death generally results within 4 days, usually exhibit a marked generalized analgesia, both superficial and deep.

Gassed cats react with no obvious sign of pain to operative interferences, including laparotomy and gentle friction of the parietal peritoneum.

The analgesia develops within a few hours after gassing, and reaches its maximum in about 24 hours. With dimethylsulfate the analgesia may persist for 6 months; with chloropicrin practically normal sensitiveness has been observed 7 days after gassing.

This analgesia is considered to be caused and maintained largely by a general, low grade, tissue asphyxia which is chiefly of pulmonic origin.

EXPLANATION OF PLATES.

The blood pressure curves were recorded from the right femoral artery with a mercury manometer. The anticoagulant for the tubing was one-half saturated sodium sulfate solution.

The time marker registers 4 second intervals, and the time line represents also the line of zero pressure.

The broad white bands on the line below the time record indicate the time and duration of nerve stimulation.

l.p.v. indicates left peripheral vagosympathetic trunk; *l.c.v.*, left central vagosympathetic trunk.

Electrical stimulation: Petzold induction coil with two Daniell cells, platinum point cable electrodes; numbers refer to distance in millimeters between primary and secondary coils.

PLATE 4.

FIG. 1. Gray and white cat, male; weight 3,760 gm. Gassed with dimethylsulfate 4.5 to 10,000 for 1 hour. Tracing obtained 26 hours after gassing. Tracheotomy performed. Left vagosympathetic nerve cut during experiment; right intact. In this cat the abdominal cavity was later opened and the parietal peritoneum gently rubbed without causing any pain.

PLATE 5.

FIG. 2. Maltese cat, female; weight 3,180 gm. Gassed with chloropicrin 1 to 10,000 for 30 minutes. Tracing made 23 hours after gassing. Tracheotomy necessary because of pulmonary edema. Left vagosympathetic nerve severed before record was taken. Slight rolling motions at the beginning of the record are betrayed by the two moderate pressure elevations near the beginning of the curve. Note very slight effect of rubbing the parietal peritoneum for 12 seconds towards the end of the tracing.

PLATE 6.

FIG. 3. White and gray cat, male; weight 4,250 gm. Gassed with dimethylsulfate 4 to 10,000 for 1 hour. Tracing obtained 6 months later; weight at this time 2,890 gm. Both vagi were intact; no tracheotomy was necessary. Opening of the peritoneal cavity and insertion of a finger caused only a slight general movement associated with a rise of 20 mm. in blood pressure. The blood pressure, pulse rate, and respiratory rate were the same at the end of the tracing as at the beginning: 130 mm., 165 heart beats, and 30 to 32 respirations per minute.

EXPERIMENTAL STUDIES ON THE ETIOLOGY OF TYPHUS FEVER.

II. SURVIVAL OF THE VIRUS IN AEROBIC AND ANAEROBIC CULTURE MEDIA.

BY PETER K. OLITSKY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 9, 1921.)

In the first paper of this series[1] it was shown that the typhus virus, in inducing the typical experimental disease in guinea pigs, readily invites invasion in the bodies of these animals of a number of bacteria, which complicate the typhus infection but, on the other hand, have no etiological relationship to the disease.

We thought to continue, in attacking the problem of the etiology of this disease, by means of a deductive method; that is, to put the virus to a variety of experimental tests so as to study the nature of any inciting agent which may reside therein. One of these tests will be described in this paper. The test concerns the survival of the virus in different media, with especial reference to the influence of anaerobic conditions. From the results obtained, we hoped to acquire particular facts which might serve as guides for cultivation experiments.

Method.

The following method was employed. The medium to be tested, under aerobic and anaerobic conditions, was inoculated with the virus present in the blood of a guinea pig reacting to experimental typhus fever. After keeping the mixture of medium and blood containing the virus for varying intervals at 37°C., the blood was removed and injected intraperitoneally into guinea pigs to determine the viability of the virus. Its activity was demonstrable when these guinea pigs developed typical experimental typhus fever, characterized by

---

[1] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 525.

(*a*) the transmissibility of the disease from animal to animal, (*b*) the presence of characteristic vascular lesions, especially in the brain, (*c*) the absence of secondary invasions with ordinary bacteria, and (*d*) the development in recovered animals of immunity to subsequent injections of typhus virus.[2] The virus was determined to be inactive when guinea pigs injected with it failed to develop either the typical experimental disease, or immunity to further injections of typhus virus.

The details of the methods of procedure were as follows:

Virus.—The virus for the following experiments was obtained from the blood of guinea pigs during the height of their reaction to experimental typhus fever. The blood was secured by cardiac puncture[3] with a sterile needle and syringe washed in 50 per cent sodium citrate solution. Three strains of virus, described in another communication,[2] were employed: human and louse strains originally derived from Poland and a human strain obtained from the blood of a Czecho-Slovakian immigrant arriving at the Port of New York.

Media.—The media used in the following tests comprised: normal rabbit plasma undiluted; normal horse serum diluted one part to three parts of saline solution; veal infusion 2 per cent peptone broth of a pH of 7.4; Smith-Noguchi tissue, ascitic fluid, sealed medium, prepared in a manner described in another paper.[4] The latter was modified as follows: in one set of experiments, omission of the tissue; in another, of the petrolatum seal; and finally, human ascitic fluid, undiluted.

Production of Anaerobic Conditions.—Comparative experiments were performed with each medium under aerobic and anaerobic conditions. An anaerobic state was produced by overlaying the medium, immediately after inoculation, with melted petrolatum. It has already been demonstrated [4,5] that this seal produces an effective anaerobic condition in a few hours in the medium underneath.

Procedure.—4 cc. of guinea pig blood, corresponding to the typhus virus, were inoculated into each of two tubes containing an equal amount of the medium to be tested. The presence of the citrate defibrinated the blood and consequently the blood settled to the bottom of the tube. One tube of the inoculated medium was left unsealed, another was overlaid with melted petrolatum (2.5 cc.). Both tubes were then kept in the same incubator at 37°C. for an indicated period of time. To test the activity of the blood, 3 cc. of the supernatant medium were removed, without disturbing the sedimented blood, and the remaining 5 cc. were injected intraperitoneally into a guinea pig. The blood in the aerobic and in the anaerobic medium was injected at the same time, so as to avoid an experimental error. To avoid further error, the experiments were repeated. For similar reasons, guinea pigs which failed to show the typical reaction after injection were observed for a period of about 1 month before they were submitted to an immunity test.

---

[2] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 365.

[3] Experiments were performed upon animals under deep chloroform anesthesia.

[4] Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

[5] Hall, I. C., *J. Bact.*, 1921, vi, 1.

RESULTS.

Virus without Addition of Medium.—As a control to aid in the interpretation of the experiments, 4 cc. of active blood, obtained from guinea pigs during the height of reaction to experimental typhus fever, were kept in a test-tube at 37°C. without the addition of any medium. The blood was kept in the incubator for 24, 48, and 72 hours. At the end of these periods, it was injected intraperitoneally into guinea pigs. Only the blood which was exposed to 37°C. for 24 hours induced typical experimental typhus fever; exposure beyond 24 hours failed to set up either the disease, or immunity to subsequent injections of the virus.

Virus Added to Broth.—Under aerobic conditions, the virus in the blood was active after remaining in this medium for periods ranging up to 5 days. After the 5th day, the virus failed to induce either the experimental disease or immunity.

Under anaerobic conditions, the virus was active after remaining in this medium for 24 and 48 hours, but failed to induce either the disease or immunity after periods longer than 48 hours.

Virus Added to Normal Rabbit Plasma.—Under aerobic conditions, the virus in the blood survived in this medium for 5 days, but failed to induce the disease or immunity in guinea pigs after longer periods.

Under anaerobic conditions, the virus survived for 24 hours, but not thereafter.

Virus Added to Normal Horse Serum.—Under aerobic conditions, the virus in the blood survived in this medium for 5 days but not thereafter.

Under anaerobic conditions, the virus survived for 24 hours, but not for longer periods.

Virus Added to Human Ascitic Fluid Media.—Under aerobic conditions, in human ascitic fluid media with or without the addition of a fragment of fresh rabbit kidney tissue, the typhus virus survived for periods of 48 and 72 hours at 37°C., but lost its activity after longer periods of exposure.

Under anaerobic conditions, in human ascitic fluid medium without rabbit kidney tissue, the virus remained viable for a period of 48 hours, but not after a longer period. In human ascitic fluid medium, to which was added a fragment of sterile rabbit kidney tissue, and which was then sealed with petrolatum—identical with the anaerobic Smith-Noguchi medium—the virus remained active for only 24 hours. In this medium after 2, 3, 4, and 5 days, the virus failed to induce either the disease or immunity to further injections of typhus virus. The addition of dextrose broth to this anaerobic medium did not prolong the period of viability of the virus.

TABLE I.

Effect of Different Media, under Aerobic and Anaerobic Conditions, on Typhus Virus.

Medium.	Condition of oxygenation.	Period of survival of virus.
		days
Broth.	Aerobic.	5
	Anaerobic.	2
Normal rabbit plasma.	Aerobic.	5
	Anaerobic.	1
Diluted normal horse serum.	Aerobic.	5
	Anaerobic.	1
Human ascitic fluid (with or without rabbit kidney).	Aerobic.	3
	Anaerobic.	2
Smith-Noguchi medium.	"	1

Table I summarizes these results.

These experiments show that the typhus virus, as it is found in the blood of guinea pigs reacting to the experimental disease, if kept at 37°C. in different media from which oxygen is excluded by a petrolatum seal, tends to die off rapidly, in 24 to 48 hours. On the other hand, the period of survival is prolonged to 5 days (in only one case to 3 days) when the same media have free access to atmospheric oxygen.

DISCUSSION AND SUMMARY.

The results of the foregoing experiments show that the typhus virus, found in the blood of guinea pigs during the height of typical experimental typhus fever, does not survive at 37°C. in anaerobic media for as long a period as in the same media under aerobic conditions. In media from which oxygen is excluded, the viability period is 24 to 48 hours; in the same media having no barrier to atmospheric oxygen, the period is usually 5 days, in one instance, 3 days.

The dead virus fails to induce not only the typical experimental disease but also an immunity to further injections of typhus virus.

That the death of the virus is due to exclusion of oxygen from the medium, rather than to a change in the hydrogen ion concentration therein is inferred from the fact that media with varying hydrogen ion concentrations, such as broth (pH 7.4), horse serum (pH 7.8), and human ascitic fluid (pH 8.0) have the same comparative effect on the virus, when under aerobic and anaerobic conditions. That is, in all, the anaerobic state causes a shortening of the viability period of the typhus virus.

In the Smith-Noguchi tissue, ascitic fluid, sealed medium in which bacteria resembling Plotz' bacilli grow luxuriantly and remain viable for several weeks, the typhus virus does not increase in virulence, and even dies after 24 hours. This evidence supports the conclusion previously presented[1] that the *Bacillus typhi exanthematici* of Plotz is not identical with the active agent of typhus virus.

CONCLUSION.

In a variety of media kept at 37°C., and from which oxygen is excluded by a petrolatum seal, the typhus virus tends to die rapidly. In the corresponding media under aerobic conditions, the life of the virus is appreciably longer.

EXPERIMENTAL STUDIES ON THE ETIOLOGY OF TYPHUS FEVER.

III. FILTRATION EXPERIMENTS.

BY PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 4, 1921.)

In a previous paper[1] we presented a plan of studying the problem of the inciting agent of typhus fever by the deductive method; that is, by applying to the typhus virus a variety of known tests in order to determine the nature of the particular incitant which may reside therein. From the experiments performed, it was concluded that the virus, in inducing experimental typhus fever in guinea pigs, invites invasion of the body of the animals by a variety of bacteria, which complicate the infection but have no etiological relationship to typhus,[2] and that the virus itself survives for a longer period in aerobic than in anaerobic culture media.[1]

In this article, the deductive method is continued and additional experiments on the typhus virus are presented relating to the filterability of the virus as it exists in the organs of guinea pigs during the height of the reaction to inoculation.

HISTORICAL.

Heretofore, most of the experiments on the filterability of typhus virus have been made with the blood taken from guinea pigs or monkeys at the height of the experimental typhus infection. Goldberger and Anderson,[3] after reviewing all previous experiments, state that there have been eight attempts, by different workers, to pass the virus of typhus through a Berkefeld filter. Of these, six were negative; in one of the other two (Wilder, 1911[4]), the monkey that had been inoculated with the filtrate, without giving any evidence of infection, was later found to be resistant to an immunity test with active blood; in the other (Nicolle, Conor, and Conseil, 1910[5]), one of two monkeys inoculated with filtrate is described as having presented a doubtful reaction, and later was found to be resistant to an inoculation with active blood.[6] Goldberger and Anderson then described their own experiments, which convinced them that the virus, as it exists in the blood, is not filterable.

---

[1] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 115.

[2] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 525.

[3] Goldberger, J., and Anderson, J. F., *Bull. Hyg. Lab., U. S. P. H., No. 86*, 1912, 62.

[4] Wilder, R. M., *J. Infect. Dis.*, 1911, ix, 9.

Later, in 1914, Nicolle, Blanc, and Conseil[7] inoculated into monkeys filtrates prepared from lice harboring the virus, and while no typical reaction resulted, it was found that on reinjection with active blood only a delayed and mild reaction occurred, which these workers regarded as evidence of a partial immunity.

In 1917, the writer's[8] efforts to filter the virus in the blood of experimentally infected guinea pigs during the reaction were fruitless. He concluded that the virus was not filterable.

The foregoing review shows that as yet no conclusive experimental basis has been offered to prove that in typhus blood the virus is filterable, although Nicolle and his associates hold that opinion.

In 1911, Nicolle, Conor, and Conseil[9] assumed that the virus of typhus fever is an intracellular parasite. Later observers, especially those who have linked the *Rickettsia prowazeki* with the incitant of typhus fever (da Rocha-Lima,[10] Wolbach and Todd,[11] and others) have taken the same stand.

The experiments to be described relate to two points: first, the supposed intracellular nature of the inciting agent, and second, the filterability of the virus. In order to approach more nearly ideal conditions of experiment, we employed organs derived from the infected animals instead of the blood.

Methods.

The spleen and brain of typhus-infected guinea pigs at the height of their reaction were chosen. Two strains of virus from human sources were used: one originating at Warsaw,[12] the other obtained from a Czecho-Slovakian immigrant at the Port of New York. Both strains have been described in a previous report.[13] That the spleen and brain are capable of inducing the experimental disease in guinea pigs has been shown by Nicolle and Blaizot[14] as well as by later workers. Indeed, Landsteiner and Hausmann[15] have induced experimental typhus fever in guinea pigs by inoculating as small a dose as 0.005 gm. of the infected brain tissue.

---

[5] Nicolle, C., Conor, A., and Conseil, E., *Compt. rend. Acad.*, 1910, cli, 685.

[6] In a later communication, we shall present evidence to show that the production of immunity in isolated instances after the injection of filtrates may possibly be dependent on other factors than a living multiplying agent.

[7] Nicolle, C., Blanc, G., and Conseil, E., *Arch. Inst. Pasteur Tunis*, 1914, ix, 84.

[8] Olitsky, P. K., *J. Infect. Dis.*, 1917, xx, 349.

[9] Nicolle, C., Conor, A., and Conseil, E., *Compt. rend. Acad.*, 1911, cliii, 578.

[10] da Rocha-Lima, H., *Arch. Schiffs- u. Tropen-Hyg.*, 1916, xx, 17.

[11] Wolbach, S. B., and Todd, J. L., *Ann. Inst. Pasteur*, 1920, xxxiv, 153.

The first experiments refer to the problem of the supposed intracellular location of the virus and to an attempt to free it through cellular disintegration.

Repeated Freezing and Thawing.—An infected guinea pig, at the height of its reaction, was exsanguinated.[16] The spleen was removed under sterile conditions; one-fifth was kept for a control test and the remainder finely pulped with scissors. The pulp was placed in a test-tube containing 20 cc. of Ringer's solution, and frozen solid by means of calcium chloride in cracked ice at −26°C. The frozen mass was then thawed out to the original fluid condition and immediately refrozen. A small portion of the pulp was removed and tested for growth *in vitro* with negative results.[17] In the meanwhile, the remainder of the pulp in suspension, with the exception of 2 cc. employed in the control test, was filtered through a tested Berkefeld V or N candle and the filtrate inoculated in the manner to be described.

Freezing and Desiccating.—In this test the spleen was frozen and then desiccated to a flaky powder according to the method of Swift.[18] As in the case of the previous method, controls of tissue before desiccation were employed. The remaining four-fifths of the desiccated splenic tissue was suspended in Ringer's solution and filtered after 4 cc. had been removed for control purposes.

Crushing in a Mechanical Tissue Crusher.—The same conditions existed in this test as in the above, except that the spleen was placed in a mechanical tissue crusher and the tissue juice and very fine homogeneous semifluid pulp resulting were suspended in Ringer's solution and filtered, the 2 cc. of the original unfiltered material being withheld for control purposes.

---

[12] We owe this strain to the kindness of Dr. S. B. Wolbach.

[13] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 365.

[14] Nicolle, C., and Blaizot, L., *Arch. Inst. Pasteur Tunis*, 1916, ix, 127.

[15] Landsteiner, K., and Hausmann, W., *Med. Klin.*, 1918, xiv, 515.

[16] All operations were performed under deep chloroform anesthesia.

[17] This test was performed by Dr. Alexis Carrel.

[18] Swift, H. F., *J. Exp. Med.*, 1921, xxxiii, 69.

Grinding with Sand.—In the same way, the cut up fragments of spleen were ground with sterile fine white sand in a mortar, with the addition, from time to time, of 5 cc. of Ringer's solution until the required amount (20 cc.) was obtained. All but 2 cc. of the thin, even, homogeneous suspension was then filtered.

Precisely similar procedures were carried out with brain tissue from infected guinea pigs.

Of the four methods employed in treating the cellular elements of the tissues, those of repeated freezing and thawing at – 26°C., and freezing and desiccating are known to kill the cells completely.[19] Crushing or grinding tissues destroys the cells, but it is presumed that an occasional living cell may escape. However, the following experiments demonstrate that the virus in the tissues prepared by any of these methods retains an undiminished infecting power. Hence one method serves to control the others.

There were thus available for testing (*a*) typhus-infected tissues of guinea pig spleen and brain, (*b*) the same tissues, either frozen, desiccated, crushed, or ground, and suspended in Ringer's solution, and (*c*) filtrates of these suspensions which were freed from the tissue cells and detritus.

Procedure.—Each experiment comprised six animals. Guinea Pig A was injected intraperitoneally with 2 cc. of the suspension of infected tissue. This animal served as a control to test the infecting power of the virus in the tissue.

Guinea Pig B was injected intraperitoneally with 2 cc. of the suspension of the unfiltered suspensions of infected tissue, either frozen, desiccated, crushed, or ground, in the order of the tests made. This animal served as a control to test the effect on the virus of the methods used in disintegrating the tissue cells.

Guinea Pigs C and D were injected with 5 cc. of the filtrate of the particular suspension of the tissue (either frozen, desiccated, crushed, or ground) to be tested. Of these animals, Guinea Pig C was allowed to run its course so as to detect any delayed reaction and, in certain instances, was reinjected, after a suitable interval, with active typhus virus in order to test its immunity. Guinea Pig D was killed from 4 to 7 days after injection,[20] and 3 cc. of its defibrinated blood were inoculated into each of two normal animals, E and F. In this way the possibility of transmission of a living, multiplying agent might be detected and the pathological changes in the body of Guinea Pig D studied.

---

[19] For the literature on this subject, see Wells, H. G., Chemical pathology, Philadelphia, 4th edition, 1920, 373.

[20] During this period the blood of guinea pigs experimentally infected contains active virus.[2]

RESULTS.

In all, fourteen experiments, as detailed above, were made with infected spleen and brain derived from guinea pigs at the height of the experimental infection: Three were made with the frozen infected tissue; two with the desiccated tissue; three with the crushed tissue, and six with the ground tissue. The results of the different experiments were practically identical.

Guinea pigs of Series A, injected with the infected tissue which was not disintegrated, showed, after an incubation of from 5 to 10 days, typical experimental typhus fever. On transmission by means of the blood obtained during the height of the reaction to normal animals, the latter, in turn, exhibited the typical fever. This was characterized by the specific histopathological picture, immunity to subsequent injections of active virus, freedom from concurrent or secondary infections, and transmissibility of the fever to normal animals—thus agreeing with the definition of the experimental disease described elsewhere.[13] Hence the materials employed for the filtration tests were proved to have specific infecting power.

Guinea pigs of Series B, injected with the infected tissue which had been either frozen, desiccated, crushed, or ground, exhibited the typical experimental typhus fever paralleling that shown by the animals of Series A.[21] Therefore, the tissue cells killed by repeated freezing and thawing, or by freezing and desiccating, or crushed into detritus by mechanical means, or ground into a homogeneous fine pulp, have had no effect on the infecting power of the typhus virus.

Guinea pigs of Series C and D, inoculated with the filtrates of suspensions of the frozen, desiccated, crushed, or ground infected tissue, failed to show the typical experimental disease. In five experiments, the animals of Series C were reinjected with active typhus virus; all failed to show any immunity. In ten experiments, the blood of guinea pigs of Series D, obtained 4 to 7 days after injection with the filtrates, was inoculated into normal animals (Series E and F). In none of these was there induced either typical experimental typhus fever or immunity. Hence the filtrates failed both to cause the typical disease and to produce immunity.

---

[21] The unfiltered desiccated tissues were injected 6 days after drying; in the instances in which the tissues were disintegrated by the other methods, the injections were made immediately after disintegration.

To summarize the results of these experiments, we find that fourteen attempts to filter the active typhus virus present in the brain or spleen of guinea pigs at the height of reaction have ended in failure.

SUMMARY AND CONCLUSIONS.

We have presented experiments to show that the typhus virus in the tissues of the guinea pig during the height of reaction to the experimental disease does not lose its infecting power when the cells of the brain or of the spleen are disintegrated by repeated freezing and thawing, or by freezing and desiccating, or by crushing by mechanical means, or by grinding into a homogeneous pulp with sand. The virus after such treatment is as actively infective as in the same tissue not subjected to the disintegrating influences. The possibility exists, therefore, of an extracellular condition of the typhus virus.

Fourteen attempts to filter through Berkefeld V and N candles the virus contained in the disintegrated tissue have all resulted in failure.

DO SPECIES LACKING A GALL BLADDER POSSESS ITS FUNCTIONAL EQUIVALENT?

BY PHILIP D. McMASTER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 23, 1921.)

It is a singular fact that certain closely related species of animals have, some of them, a gall bladder, while others do not. What can be the essential significance of the organ and the reason for this difference?

Embryologically, the vesica fellea arises secondarily as a cul-de-sac from the same anlage that forms the liver and bile ducts, and it is considered by many as an almost purposeless diverticulum. The erroneous nature of this view is sufficiently shown by the several functions now demonstrated for the organ. The matter has been discussed in a previous paper from this laboratory.[1] The fact that the gall bladder is a highly specialized organ renders all the more noteworthy its irregular distribution in both high and low forms of life. Thus among the higher animals it is present in the cow and sheep, while it is absent in the horse, present in the goat, and absent in the closely related deer—to be found in the hog and wild boar but not in the peccary of South America. Among birds, the hawk and owl possess it, while doves do not; and among the rodents the mouse is found with the organ, the rat without. One species of gopher,[2] the pocket gopher (*Geomys bursarius*), is without a gall bladder, while another (*Spermophilus tredecimlineatus*) the striped gopher, possesses it. Woods Hutchinson[3] is authority for the statement that in the giraffe it is at times present and again not.

---

[1] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

[2] Mann, F. C., *J. Lab. and Clin. Med.*, 1919–20, v, 107.

[3] Hutchinson, Woods, *Med. Rec.*, 1903, lxiii, 770.

The disposition of the bile ducts or the position of the pancreas throws no light on the riddle of the gall bladder. If we consider only animals without the organ, the horse has separate pancreatic and common bile ducts emptying into the duodenum through a common opening. The deer, rat, and pocket gopher (*Geomys bursarius*) show the pancreatic duct opening directly into the common bile duct well above the ampulla, this it also does in both rats and white mice, the former being without a gall bladder, the latter with it. No inference can be drawn from the high opening of the pancreatic duct, for this is as inconstant[4] as the presence of the gall bladder itself.

The vesica fellea has, as already indicated, functions sharply different from those of the ducts. Its rôle as a reservoir may on occasion, as after cholecystectomy, be partially taken over by the ducts.[5] Not so, however, with its modifying influences upon the bile. The bladder acts to concentrate the secretion greatly and to thicken it with mucus, whereas the ducts by contrast tend to dilute it, though to a negligible degree, with a thin product of their own.[6] Is it possible that in animals lacking the gall bladder, the concentrating function is lodged somewhere in the duct wall? This possibility can readily be tested out by ligation experiments on rats and mice.

If the gall bladder has the same activities in the mouse as in the dog, cat, and monkey,[1] then the bile submitted to its influence after ligation of the common duct should become concentrated by the action of the gall bladder. Granting that this really occurs,—and the findings now to be described show that it does,—will a concentration of bile also go on in the obstructed duct system of the rat, an animal devoid of a gall bladder, or will this bile be diluted as is the case with bile pent in the ducts of other animals which have been deprived of their connection with the bladder? The close relationship of the species and their similarity in habits give a special value to the test.

[4] Mann, F. C., Foster, J. P., and Brimhall, S. D., *J. Lab. and Clin. Med.*, 1919–20, v, 203.

[5] Haberer, H., and Clairmont, P., *Verhandl. deutsch. Ges. Chir.*, 1904, xxxiii, pt. 2, 81. Rost, F., *Mitt. Grenzgeb. Med. u. Chir.*, 1913, xxvi, 710.

[6] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 75.

Method.

The pigment content of the bile was taken as the criterion of extrahepatic changes in concentration. Its utility in this connection has already been shown.[1] The range of the normal pigment content of mouse and rat bile was first determined with the animals on their ordinary ration (bread and milk, and grain).

From rats the bile was obtained into small rubber bags connected with fine cannulas inserted in the common duct. The bags were left within the animal's peritoneal cavity for 24 hours or more. Considerable amounts of secretion, ample for pigment analyses, were readily obtained in this way. The small size of the mouse, however, made necessary a different method, which will later be described.

All operations were carried out under ether, with aseptic precautions. To produce stasis, a triple ligation of the duct was done, the ligatures being placed just below the junction of the lowest hepatic duct with the common duct and well above the pancreatic duct entrance, and the common duct was then cut between the middle and lowest ties. The animals were allowed to live thereafter for periods of 1 to 16 days on a mixed diet of barley and oats, with bread and milk. Recovery from the operative procedure was rapid and the animals ate well. They were killed at intervals, the stasis bile was collected into a pipette, and its pigment content determined. In no instance had the continuity of the duct been reestablished. Cultures of the bile and liver tissue were regularly taken.

The Quantitation of Pigment.

Different methods were used to determine the pigment content of bile according to the amount available. When more than 0.3 or 0.5 cc. of rat bile was had, a reading was carried out according to the colorimetric method of Hooper and Whipple.[7] To a measured quantity of bile is added a known quantity of acid alcohol reagent. For a standard, instead of a wedge of artificial constitution, a chloroform solution of pure bilirubin (Schuchardt) was used, which was mixed with the reagent, as was the bile, and read against it in a Duboscq colorimeter.

When the quantity of rat bile was very small, the mixture of it with the acid alcohol reagent was made in a Miescher pipette such as is used in the Fleischl-Miescher hemoglobinometer and read against the standard bilirubin solution as ordinarily, but in a micro colorimeter.

---

[7] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

The pigment content of normal mouse bile in the small quantities obtained was always insufficient to yield, with the reagent, a green color that could be read in the colorimeter. It was quantitated directly against a potassium bichromate scale, a method used as well with rat biles. For the purpose, glass tubes with a bore of 0.75 mm. and walls so thick (2 mm.) as to result in a magnification, were filled with bile and with the scale solutions. The tint of bile and standard matched very closely. A permanent series of tubes was made containing dilutions of potassium bichromate ranging from 6.0 to 0.05 per cent; and tests were made to determine whether the color intensity of mouse bile varied on dilution like that of a weak bichromate solution. Specimens of bile which corresponded in color with bichromate solutions of known percentage were diluted and compared again with the scale. They now agreed with a percentage bichromate solution corresponding to their dilution. Thus a bile solution corresponding in color with a 1 per cent bichromate solution, when diluted with equal parts of water, had the color strength of a 0.5 per cent bichromate solution—when with ten parts of water it corresponded with a 0.1 per cent solution, and so on. In order to standardize the scale, a number of normal rat biles of known pigment content, taken at random, were compared with the bichromate tubes. The results of this comparison have been assembled in Table I. There three subdivisions have been made according to the pigment strength of the bile employed. The bilirubin content in milligrams per 100 cc., as determined by the acid alcohol reagent, is given in Column A, and its reading in percentage of bichromate solution, as determined by comparison with the small glass tubes, is given in Column B. If the relative amount of pigment can be read as truly on the bichromate scale as by the Hooper and Whipple method, then $\frac{A}{B}$ should yield a constant, which constant incidentally is the expression in milligrams of bilirubin per 100 cc. of bile, of the colorimetric value of a 0.1 per cent bichromate solution, or for a 1 per cent solution, when multiplied by 10. It will be seen that the constant was only approximately yielded by the individual bile specimens, but the variation is much the same throughout the groups, and the average for the constant was 13.26, 13.51, and 13.57 for biles of

TABLE I.

Standardization of the Potassium Bichromate Scale.

Rat No.	A Actual amount of bilirubin per 100 cc.	B Per cent strength of bichromate solution corresponding in color to the bile.	$\frac{A}{B} \times 10$	Averages.	
	mg.				
8	8.9	0.7	12.7	Pigment less than 15 mg. per 100 cc. of bile. Average 13.26	13.45
37	11.7	0.8	14.6		
38	12.5	0.9	13.77		
9	14.38	1.2	11.98		
7	15.43	1.4	11.02	Pigment amounts 15 to 20 mg. per 100 cc. of bile. Average 13.51	
6	16.8	1.4	12.0		
14	17.85	1.2	14.88		
15	19.1	1.2	15.91		
10	19.23	1.4	13.75		
5	20.16	1.8	11.2	Pigment amounts above 20 mg. per 100 cc. of bile. Average 13.57	
16	20.83	1.5	13.88		
13	22.32	1.6	13.9		
11	22.72	1.5	15.15		
12	24.51	1.8	13.6		

All bile collections were made by the bag-cannula method.

Rat biles collected by the bag-cannula method were employed. In Column A, the actual milligrams of pigment per 100 cc. of bile, as obtained by the acid alcohol method, are given. Column B gives the pigment strength of the bile in per cent of potassium bichromate solution as obtained by direct color comparison. If this latter comparison is a true one, then $\frac{A}{B}$ should yield an expression in milligrams of bilirubin per 100 cc. of bile of the colorimetric value of a 0.1 per cent bichromate solution, as determined from that particular specimen of rat bile. This multiplied by 10 should give the figure for a 1 per cent solution ($\frac{A}{B} \times 10$), and should be a constant. The readings are separated into three groups according to whether the pigment content of the bile fell below 15 mg. per 100 cc., between 15 and 20 mg., and over 20 mg. per 100 cc. It will be seen that the same approximate constant ($\frac{A}{B} \times 10$) is yielded by all three groups.

low, medium, and high pigment content respectively, the general average being 13.45. As the table shows, a bile which reads on the bichromate scale at 1 per cent will contain about 13.45 mg. per 100 cc. Thus a specimen of rat or mouse bile obtained from a duct could be placed in one of the small bore tubes, compared with the bichromate scale, and its pigment content quantitatively estimated.

The Pigment in Normal Mouse Bile.

The delicacy of the common duct in mice prohibited the use of the bag and cannula method which was successful with rats. Instead, bile was collected from animals under ether, through extremely fine long cannulas, with the aid of suction.

To obtain cannulas of sufficient flexibility, hard glass tubing was drawn out into capillary pipettes with an external diameter of about ½ mm. and a length of 40 to 60 cm., with the small end carefully rounded to avoid laceration of the duct. The larger end of the tube was curved and the bend in it formed a sort of basin into which the bile from the capillary collected. Mild suction was maintained at this end, when desired, through a connection with a rubber tube and bulb filled with water, which could be lowered at will.

The larger end of the cannula was rigidly attached to a mechanical stage which permitted accurate movement in two dimensions of space and its flexible shaft was passed through three fixed wire loops, so arranged as to give it a marked downward curvature. All of the loops could be raised or lowered at will, thus increasing or decreasing the curvature of the shaft and correspondingly deflecting the open end of the cannula, so that it could be made to take the exact direction of the bile duct in the individual mouse. This adjustment was essential to prevent kinking and obstruction of the duct.

Under ether, the bile duct was exposed close to its entrance into the duodenum. By the use of the mechanical stage the cannula was then introduced either through a slit in the duct or by ligating the duodenum to either side of the papilla of Vater, opening it and thrusting the cannula through the papilla into the duct. A magnifying glass placed above the field of operation was found of service. The cannula once in the duct was always advanced to a point well above the entrance of the channel and in many instances tied in place, thus blocking off all pancreatic secretion. Often it plugged the duct so tightly that this was unnecessary. Mild suction was produced on the capillary pipette and the animal kept under ether for several hours while the flow of bile went on.

By this arrangement the gall bladder was left in direct connection with the cannula and *a priori* one would suppose that its contents formed part of the normal bile collected through this latter. However, on first opening the abdomen to insert the cannula, the gall bladder was usually noted to be empty. Even when full, little of its contents was yielded to the cannula on suction.

Collections were successfully made by this method for periods as long as 6½ hours. Body warmth was kept up by electric lights placed near the animal, and injury was combated with moist sponges. The bile obtained was very uniform in character, exceedingly abundant, clear, light amber-yellow but, as already said, too weak in pigment, in the small quantities obtainable, to yield a notable blue-green color with the acid alcohol reagent.

Table II shows the result of readings against the bichromate scale with the estimated amounts of bilirubin per 100 cc. of bile. An average from ten cases gives a pigment content of 2.17 mg. of bilirubin per 100 cc. of bile. The quantity of bile secreted per 100 gm. of body weight in an hour was about 0.2 cc., or, per kilo of body weight per day (24 hours), 34.0 cc. A gram of liver tissue of the mouse secreted about 0.03 cc. of bile per hour.

Results of Obstruction in Mice.

The bile was studied from many mice in which obstruction had been produced by ligation of the common duct. Obstruction lasting 1 to 3 days did not result in a marked dilatation of the ducts but the gall bladder was always found full of a bile much darker and more mucinous than normal mouse bile. It had early a clear, dark amber color, and the longer the obstruction the darker the bile. After 3 days, the ducts became dilated, though the gall bladder did not greatly increase in size. Both now contained abnormally dark bile in greater and greater amount from day to day. The actual quantity was difficult to measure, since in puncturing the ducts some was almost always lost.

In accordance with expectations based on the findings in larger animals with gall bladders,[1] the stasis bile always contained much more than the normal amount of bilirubin (Table III). When stasis had existed for more than 24 hours, the gall bladder bile had sometimes a dark greenish tint. This change of some of the pigment to biliverdin caused difficulty in the comparison with the bichromate scale. Fortunately, the pigment amount was so great as to yield, even in the small quantities of bile at hand, the acid alcohol reaction of Hooper and Whipple whereby biliverdin and bilirubin are quantitated together.

Table III shows that the bile obtained after 1 day of stasis ranged in color strength, as expressed in terms of the bichromate scale, from 1.5 per cent bichromate to 4 per cent, with an average of 2.25 per cent. In normal animals, by contrast, (Table II) the range was from 0.1 to 0.3, and averaged 0.16 per cent. The stasis bile, then, was fourteen times as strong in pigment as the normal. After 1 day of stasis, no perceptible conversion of bilirubin to biliverdin had occurred.

TABLE II.

Pigment Content of Normal Mouse Biles.

Mouse No.	Mouse weight.	Liver weight.	Period of collection.	Total bile.	Quantity of bile per 100 gm. of body weight per hr.	Quantity of bile estimated per kilo of body weight per day.	Quantity of bile per gm. of liver per hr.	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimated from reading of bichromate scale.
	gm.	*gm.*		*cc.*	*cc.*	*cc.*	*cc.*		*mg.*
1	23.0	1.15	2 hrs., 45 min.	0.1+	0.158	38.0	0.032	—	—
2	21.6	1.4	4 " 15 "	0.06 (incomplete).	0.065	15.6	0.011	0.1	1.35
3	20.0	1.4	1 hr., 25 "	0.15	0.53	12.7	0.075	0.1	1.35
4	24.5	1.9	4 hrs., 20 "	0.29	0.273	65.5	0.035	0.1	1.35
5	23.3	1.2	5 " 5 "	0.14	0.118	28.3	0.023	0.15	2.02
6	24.0	1.2	2 " 50 "	0.04	0.059	14.2	0.012	0.3	4.03
7	27.0	1.9	2 " 50 "	0.12	0.157	37.6	0.022	0.2	2.69
8	24.0	1.8	5 " 20 "	0.32	0.25	60.0	0.033	0.1	1.35
9	—	—	5 " 40 "	0.18	—	—	—	0.25	3.36
10	—	—	3 " 10 "	0.05 (incomplete).	—	—	—	0.15	2.02
Average					0.20	34.0	0.03	0.16	2.17

In a number of cases bile found in the gall bladder was compared with that collected from the ducts of the same animal. The pigment strength of the bladder and duct biles were compared with the aid of the bichromate scale (Table IV).

TABLE III.

Pigment Content of Mouse Biles from Obstructed Ducts.

Mouse No.	Mouse weight.	Liver weight.	Period of obstruction.	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimated from reading of bichromate scale.	Actual amount of bilirubin per 100 cc. of bile (acid alcohol method).
	gm.	*gm.*	*days*		*mg.*	*mg.*
11	27.8	2.25	1	2.0	26.89	—
12	23.0	1.7	1	4.0	53.78	52.08
13	19.5	1.2	1	2.0	26.89	—
14	24.2	2.2	1	1.5	20.17	—
15	21.5	1.35	1	2.2	29.58	26.04
16	27.75	1.55	1	—	—	82.6
17	—	—	1	—	—	47.7
18	25.4	2.1	1	2.0	26.89	27.77
19	25.5	1.85	2	—	—	—
20	23.0	1.2	2	—	—	52.1
21	25.0	1.9	2	—	—	38.1
22	—	—	13	—	—	32.0
23	—	—	14	—	—	35.0
Average				2.25 (after 1 day of obstruction only).		

Average pigment content of bile after 1 day of obstruction is 30.7 mg. per 100 cc.

TABLE IV.

Comparison of the Pigment Strength of the Bladder and Duct Biles from Mice at the Close of the Bile Collection.

Mouse No.	Results.
4	Gall bladder bile showed 7 times the concentration of duct bile after a 4 hrs. collection.
5	“ “ “ “ 4 “ “ “ “ “ “ “ “ 5 “ “
6	“ “ “ “ 3 “ “ “ “ “ “ “ “ 3 “ “
7	“ “ “ “ 5 “ “ “ “ “ “ “ “ 3 “ “
8	“ “ “ “ 5 “ “ “ “ “ “ “ “ 5⅓ “ “
9	“ “ “ “ 4 “ “ “ “ “ “ “ “ 5¾ “ “
Average.	“ “ “ “ 4.66 “ “ “ “ “ “ “ “ 4.3 “ “

Normal and Stasis Biles of the Rat.

The rubber bags used to obtain normal bile from rats were made out of finger cots and attached to glass cannulas of capillary diameter, with the ends well rounded and the shaft curved to avoid torsion on the duct. The cannula was fastened in the duct with ligatures above the entrance of the pancreatic duct, and in some instances the pancreatic duct as well was tied. Such fat necrosis as sometimes followed this latter procedure seemed not to affect the animal materially in the short period of bile collection. The rats ate well after the operation. At the end of 18 to 24 hours they were killed with chloroform.

To produce obstruction, ligature and severance of the common duct was employed. Bile leaks into the peritoneal cavity seldom were found and animals in which they occurred were discarded. Tissue icterus always appeared in 24 hours and bile pigment was plentiful in the urine. The twenty operated animals were killed at intervals ranging from 1 to 16 days after obstruction.

At autopsy the degree of dilation of the obstructed bile ducts was noted and the approximate amount of bile present within them. Very occasionally positive cultures were obtained from bile or liver tissue of the rats into which bags had been inserted, but, probably owing to the short period of collection, such biles seemed otherwise normal. The not infrequent infected cases among the animals with obstruction were ruled out. The collecting bags gave an abundant yield of bile—never less than 1.5 cc. and as much as 6.5 cc. per 100 gm. of body weight in 24 hours.

From Table V it will be seen that the pigment content of normal rat bile as determined in sixteen animals was 17.05 mg. of bilirubin per 100 cc., with a variation of 8.9 to 22.3 mg. It is of interest to note that the bulk of bile secreted per gram of liver tissue per hour in rats and mice is almost identical, but the proportion of liver weight to body weight in rats, 1 to 21.7, is considerably smaller than that of mice, 1 to 14.6. The quantity of bile secreted per 100 gm. of body weight was, per hour, 0.151 cc., or per kilo of body weight per day (24 hours) 36.3 cc.; and of bile per gram of liver per hour 0.034 cc., or per day 0.82 cc.

The normal rat bile was always a clear amber fluid as viewed in a test-tube, quite transparent, and a brilliant light yellow when placed in one of the reading tubes for comparison with the bichromatic scale. It showed remarkably little variation in pigment concentration, but had as a rule relatively less color when the amount of bile

TABLE V.

Normal and Stasis Biles of the Rat.

	Pigment content of normal biles.									Pigment content of biles from obstructed ducts.						
Rat No.	Rat weight.	Liver weight.	Period of collection.	Total bile.	Quantity of bile per 100 gm. of body weight per hr.	Quantity of bile estimated per kilo of body weight per day.	Quantity of bile per gm. of liver per hr.	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimated from reading of bichromate scale.	Actual amount of bilirubin per 100 cc. of bile (acid alcohol method).	Rat No.	Rat weight.	Liver weight.	Period of obstruction.	Color strength in per cent of bichromate solution.	Amount of bile pigment per 100 cc. estimat d from reading of bichromate scale.	Actual amount of bilirubin per 100 cc. of bile (acid alcohol method).
	gm.	*gm.*	*hrs.*	*cc.*	*cc.*	*cc.*	*cc.*		*mg.*	*mg.*		*gm.*	*gm.*	*days*		*mg.*	*mg.*
1	166	8.7	25	3.6	0.087	20.8	0.017	—	—	13.87	17	109.0	6.0	1	1.3	17.48	—
2	158	7.5	25	5.7	0.144	34.5	0.030	—	—	12.33	18	99.0	5.0	1	1.2	16.14	—
3	122	7.0	22½	7.1	0.258	62.0	0.045	—	—	13.55	19	88.7	4.4	1	1.6	21.51	—
4	160	9.5	22	6.2	0.176	42.6	0.030	—	—	13.15	20	92.5	4.2	1	1.4	18.82	—
5	—	—	19	2.4	—	—	—	1.8	24.20	20.16	21	76.5	3.7	2	1.2	16.14	—
6	92	5.8	—	—	—	—	—	1.4	18.82	16.8	22	77.5	5.5	2	1.3	17.8	—
7	178	7.2	18	2.5	0.078	18.7	0.019	1.4	18.82	15.43	23	92.2	5.5	3	1.2	16.14	—
8	163	7.7	22	7.8	0.218	52.2	0.046	0.7	9.41	8.93	24	80.8	5.1	3	1.3	17.8	—
9	158	6.1	22	—	—	—	—	1.2	16.13	11.98	25	—	—	4	1.8	24.2	—
10	163	6.5	18	4.8	0.164	39.4	0.041	1.4	18.82	19.23	26	93.2	4.7	5	1.8	24.2	25.75
11	170	6.6	18	2.9	0.095	22.8	0.024	1.5	20.17	22.72	27	—	—	5	—	—	14.0
12	158	5.8	18	1.8	0.063	15.1	0.017	1.8	24.20	24.51	28	—	—	5	—	—	15.4
13	153	6.5	18	3.1	0.113	27.1	0.027	1.6	21.51	22.32	29	—	—	5	—	—	10.68
14	107	4.2	18	5.2	0.271	64.9	0.068	1.2	16.14	17.85	30	93.9	5.8	7	2.0	26.89	26.31
15	167	6.9	18	4.1	0.136	32.8	0.033	1.2	16.14	19.1	31	170.0	8.1	10	1.5	20.17	17.86
16	158	6.2	18	4.6	0.162	38.9	0.041	1.5	20.17	20.83	32	—	—	10	1.5	20.17	14.2
											33	166.2	8.0	12	0.8	10.76	13.88
											34	150.7	7.8	12	0.8	10.76	12.5
											35	—	—	12	—	—	18.4
											36	—	—	16	—	—	18.45
Average.	151.3	6.95			0.151	36.3	0.034		18.71	17.05						18.6	17.02

Proportion of liver weight to body weight in the rat is 1 to 21.7. For this average only the data of normal bile collection rats were used.

secreted was great. The stasis bile was closely similar. No change from yellow to green took place such as occurs in the dog and cat, and occasionally in mouse bile after long obstruction. Though the dilatation of the ducts became greater each day, the pigment strength of their contents was never more than that of normal bile, and as a matter of fact as time passed the concentration of pigment approximated the lowest normal figures, probably as a result of dilution with fluid elaborated by the duct wall, as in the case of dogs and cats.[6] In contrast to these results, stasis bile of the mouse always underwent a marked concentration, as will be recalled.

To find out whether there might be an early concentration of bile in the duct, followed by a later dilution as indicated, the sequence of changes in the bile after obstruction was carefully followed by means of animals killed on successive days. After the 5th day of obstruction, sufficient bile could be collected in the duct for a quantitative reading with acid alcohol, but in all cases the bichromate scale was used as with the animals killed earlier. The results in this sequence of animals are distributed amidst the earlier ones in Table V, with which they agree completely. The final average of 17.02 mg. of bilirubin per 100 cc. of bile in obstructed instances, as determined by the alcohol reagent, is remarkably similar to the figure obtained for normal bile—17.05 mg. The bichromate reading of 18.6 mg. per 100 cc. of bile also compares with the result found in non-obstructed cases of 18.7 per 100 cc. It is thus seen that rat bile undergoes no concentration after leaving the liver, even during long stasis. The secretion is about eight times as strong in pigment as mouse bile obtained from the ducts.

From these findings it is clear that there resides in the rat ducts no ability to concentrate bile such as resides in the gall bladder of the mouse. The dilatation of the rat ducts was, relatively speaking, enormous, and progressed with such regularity that one could almost state from it the number of days of obstruction. In several instances the common duct reached a diameter of 15 mm. in its broadest portion and it contained in one case more than 2.8 cc. of bile which, though still of a clear pale amber and a normal pigment content, contained more mucus than normal bile. In some animals the intrahepatic dilatation of the ducts was so great that the parenchyma had become merely a sort of covering upon them. The liver tissue showed marked cirrhosis and was clay-yellow. Histologically the condition seen was similar to that found in dogs after long obstruction—a perilobular cirrhosis.

Influence of Diet.

The amount of bile secreted by the rat (36.3 cc. per kilo of body weight per day) and by the mouse (48.3 cc.) is not nearly as great as is put out by the rabbit and guinea pig, but is more than the output of the dog and cat. Thus, according to Quincke and Hoppe-Seyler[8] the rabbit secretes 136.8 gm. of bile per kilo of body weight a day and the guinea pig 175.8 gm., while the dog yields but 20.0 gm. and the cat 14.5 gm. per kilo of body weight. The large bile output of the Herbivora has usually been ascribed to their diet; and our animals were on a vegetable ration. To determine the importance of this factor, several rats were placed on a meat diet for a period of 17 days, and bag collections were then made as in normal grain-fed animals. But the bile obtained was not unusual either in amount or character.

SUMMARY.

In a previous paper the point has been brought out that the influence of the gall bladder upon the bile differs entirely from that of the ducts, the one organ acting to concentrate the secretion markedly and the other to dilute it slightly. The question arises, in species lacking a gall bladder, whether the concentrating function of this organ will be found lodged in the ducts. To test the point, observations have been made upon the mouse and rat, since these animals though so nearly related have, the mouse, a gall bladder and the rat, none.

The normal bile was first studied. Both animals were found to secrete larger quantities than do cats and dogs, but less than the guinea pig and rabbit. Methods were worked out for the quantitation of the pigment which was used as the index to changes in concentration.

---

[8] Quincke, H., and Hoppe-Seyler, G., in Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1899, xviii, 59.

Bladder bile of the mouse was regularly found to be more concentrated than that collected from the common duct of the same animal. The bile collecting during stasis regularly showed a great increase in pigment content, such as in other species is brought about by the action of the gall bladder. In the rat, on the other hand, stasis bile never became more concentrated in pigment than the normal.

The gall bladder, then, is not only absent from the rat in form, but in one at least of its important functions. That its other obvious function—that of a reservoir—cannot be assumed in the rat by the ducts would seem to be indicated, not only by the small size of these channels, but by the recent observation of Mann[2] that the tonus of the sphincter of Oddi is almost negligible in the rat, in contradistinction to animals which possess a gall bladder.

It is an interesting fact that the bile of the rat, which as has been said, undergoes no condensation of bulk after leaving the liver, contains on the average eight times as much pigment as does the liver bile of the mouse which is submitted to concentration. Whether it is correspondingly strong in substances useful for digestion, and therefore *ab initio* requires no concentration, is a matter upon which little can be said at present. However, in this connection the fact that the bulk of bile secreted per gram of liver weight is identical in both animals may be significant. Although this output is the same, the mouse liver when compared with the body weight (1 to 14.6) is relatively larger than that of the rat (1 to 21.7), so that the mouse secretes somewhat more bile per 100 gm. of body weight. This bile as it comes from the liver is but one-eighth as strong at least in pigment as rat bile, but the concentrating activity of the gall bladder is so great that the products yielded to the intestine may become not dissimilar.

SOURCES OF THE ANTIBODIES DEVELOPING AFTER REPEATED TRANSFUSION.

By OSWALD H. ROBERTSON, M.D., AND PEYTON ROUS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 1, 1921.)

The recent wide utilization of transfusion as a therapeutic measure has brought to light many facts of theoretical as well as practical significance. Perhaps most interesting from both points of view is the gradual decrease in beneficial effect, and the appearance sometimes of positive injury from the frequently repeated injection of alien blood. Were it not for this complication one might reasonably expect to maintain cases of pernicious anemia in good blood condition for an indefinite period of time. Needless to say, the sources of the failure have been the subject of much discussion and of some research. One fact of great weight has been clearly shown. Blood derived from a donor originally compatible, as proven both by *in vitro* tests and by the clinical result, may not only cease to be useful when too frequently injected into the same individual but may give rise to serious reactions. A change has occurred, not in the donor but in the recipient, such that the alien blood is no longer tolerated in circulation.

Boycott and Douglas[1] have noted that blood is destroyed more rapidly after repeated transfusions in normal animals than it is at first, as attested by an increase in the rate at which plethora disappears. One of us, with Oliver,[2] has utilized the phenomenon to induce in rabbits a hemosiderosis closely resembling that of hemochromatosis in man. No doubt a part of the pigmentation observed at autopsy in pernicious anemia patients who have been repeatedly transfused is due to a like destruction of alien blood. But how is this blood destroyed? By circulating antibodies or within special organs?

---

[1] Boycott, A. E., and Douglas, C. G., *J. Path. and Bact.*, 1909, xiii, 414.

[2] Rous, P., and Oliver, J., *J. Exp. Med.*, 1918, xxviii, 629.

Recent observations indicate that circulating antibodies are to a considerable degree involved.[3] It has been found that repeated transfusions of compatible blood in rabbits are followed often by the appearance in the recipients' plasma of hemagglutinins so strong that the red cells come together into a firm mass practically as soon as the blood has been shed, while, furthermore, a fulminant destruction of corpuscles may take place *in vivo* with result in anemia. Robertson[4] has presented evidence that the elements destroyed are the alien cells which, little by little, under the circumstances of plethora and diminished bone marrow activity consequent thereon have taken the place of cells proper to the host.

The hemagglutinins just mentioned are in the immediate sense autoantibodies. They clump practically all of the circulating erythrocytes, are especially effective at low temperatures, and persist in high titer for months after the transfusions have been discontinued and after recovery from the severe intercurrent anemia which may develop soon after their appearance. Is it possible that an organism engaged in eliminating unusually large amounts of blood will elaborate antibodies directed against its own cells? This is a point of major interest in any study of the source of the hemagglutinins, and one not without a practical bearing. In human beings true autohemagglutinins have repeatedly been observed[5] in association with anemia of obscure origin; while autohemolysins are known to bear an important relation to paroxysmal hemoglobinuria.

Transfusion Method.

Three to six compatible donors were selected for each recipient by the examination of mixtures of the citrated bloods.[6] The recipients received, 6 days in every 7, 10 cc. of blood taken by cardiac aspiration into 1 to 2 cc. of 0.9 per cent salt solution containing 1 per cent of sodium citrate. The donors were employed in rotation. The small amount of citrate mentioned was sufficient to prevent clotting during the short period required to introduce the blood into the recipient's ear vein. About half of the transfused animals failed to develop autoagglutinins at any time, even when the injections were continued for many weeks. In the other individuals they appeared early, as a rule after only five to ten transfusions; and when three or four more had been given, that is to say, after some 10 days to 3 weeks in all, they were well marked. The shed blood of the rabbits, when examined at room temperature, now showed prior to clotting a massive clumping of the red cells due, as our previous work has shown, to the presence in the plasma of true hemagglutinins.[3]

---

[3] Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1918, xxvii, 509.

[4] Robertson, O. H., *J. Exp. Med.*, 1917, xxvi, 221.

[5] Clough, M. C., and Richter, I. M., *Bull. Johns Hopkins Hosp.*, 1918, xxix, 86.

[6] Rous, P., and Turner, J. R., *J. Am. Med. Assn.*, 1915, lxiv, 1980.

The Mixed Content of the Blood.

The transfused rabbits became, as it were, mixing vessels for several alien corpuscles and plasmas, perhaps indeed concentrators of immune principles through the elimination of the fluid wherewith the latter were introduced. The clumping of the cells of the shed blood might conceivably have been the result of:

1. The injected sodium citrate—a possibility ruled out by negative findings in control animals given citrate alone.
2. Antibodies introduced with the donor's plasma and (*a*) active against the recipient's cells. These might either have developed during the period of experiment, or have been so weak as to escape detection *in vitro*. No tests had been made to rule out (*b*) interagglutination among the donor bloods.
3. Antibodies elicited in the recipients and directed against (*a*) the strange corpuscles and (*b*) against the animal's own corpuscles.
4. Changes in the circulating cells resulting in a greater agglutinability.
5. Antibodies within the injected corpuscles, liberated by their destruction.

More remote possibilities could be invoked, as for example, a nonspecific agglutination brought about through physical changes in the plasmas pooled *in vivo*, but, as will be shown, those above listed suffice to account for the findings.

Tests with Preserved Cells.

In an initial series of experiments for the analysis of the conditions, blood specimens were taken aseptically from the individuals of several donor-recipient groups prior to any transfusions, and set aside in a citrate-glucose mixture in which rabbit corpuscles remain viable for several weeks.[7] Later when the transfusions had elicited the clumping phenomenon, these preserved corpuscles were several times washed, made to a 5 per cent suspension with salt solution, and used in agglutination tests paralleling others carried out at the same time with freshly taken cells of the same animals.

For the purpose, equal parts of cell suspension and serum were mixed, allowed to stand for 15 to 30 minutes at room temperature, and examined microscopically. Room temperature is far more favorable to hemagglutination than blood heat. Clumping of the cells is ordinarily completed in about 10 minutes.

The tests yielded clear-cut results. Prior to transfusion no agglutination reaction was ever observed between the bloods of the prospective donors and recipients, when examined either according to the method just described or by that with whole citrated bloods previously referred to. Weak interagglutinations were sometimes noted between the bloods of certain donor groups but they were entirely absent from others. The fresh and preserved cells of the same individual were regularly found to behave almost identically on test *in vitro*. No evidence was obtained for possibility No. 4 of those above listed, namely an increase in agglutinability of cells circulating in an alien organism; but several other causes for the clumping were readily demonstrated. Most important, and most frequent, were isoagglutinins, often of high titer, developing in the recipient and effective against the cells of one or more donors (possibility 3, *a*). Such antibodies commonly appeared, and were readily demonstrable in serum separated from the clot at room temperature. Their association with the most marked clumping noted makes it highly probable that the sudden anemia supervening on plethora which occurs only in association with such marked clumping is due to isoantibodies.

Agglutinins interactive between the donor bloods (possibility 2, *b*) sometimes developed, but they were never strong. Occasionally agglutinins for the recipients' cells appeared in the donors' plasma during the period of transfusion (possibility 2, *a*). These were always so weak as to be negligible *in vivo*.

There remained a series of instances in which, with all such antibodies ruled out, there were yet hemagglutinins in the recipient that led to a marked clumping not only of the mixture of cells present after transfusion but of the recipient's cells taken prior to transfusion and kept *in vitro*. Such antibodies, like true autoagglutinins,[8] were readily bound to, and completely freed from, the cells by cooling and warming respectively. Tests on their origin were now begun; and to rule out wholly the possibility that undetected antibodies had been injected with the donors' bloods, the transfusions were carried out with washed cells.

---

[7] Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

Transfusions with Washed Cells.

Five donor-recipient groups were chosen in the usual way and the donors bled in rotation. 10 cc. of whole blood was taken each time into about 20 cc. of a sterile isotonic solution of sodium citrate (3.8 per cent) in water and allowed to stand in the ice box for 2 days, during which period practically complete sedimentation occurred. The supernatant fluid was now pipetted off and the cells were suspended in about 20 cc. of Ringer's solution, to which had been added 0.125 per cent of gelatin and 0.2 per cent of sodium citrate. The gelatin prevented mechanical injury of the cells during the manipulations, and the citrate did away with the slight tendency to clotting. After a further 2 days of sedimentation in the cold the corpuscles were again suspended, but this time in gelatin-Ringer's solution lacking citrate, and immediately thrown down with the centrifuge; made to a total bulk of 10 cc. with an ordinary Ringer's mixture containing only 0.76 per cent NaCl; and injected. During all the handling no hemolysis occurred except occasionally to a negligible degree in the case of a donor with unusually frail cells.

Prior to the first transfusion, blood specimens from the prospective recipients were taken for preservation as already described.

In three of five rabbits repeatedly injected with the washed cells a well defined clumping in the shed blood was soon noted. In the two better marked cases the serum of the recipient, separated from the cells at 37°C., caused at room temperature outspoken agglutination of the preserved cells of the same individual. The principle which caused the red cells to come together remained fixed upon them in the cold, and the cell masses could be repeatedly washed in salt solution without its liberation. But when the fluid was warmed to body heat the agglutinins passed into it and the cell mass separated into its components. By such means the antibody proved readily obtainable in salt solution, as is the case with autoagglutinins.[8]

---

[8] Landsteiner, K., *Münch. med. Woch.*, 1903, l, 1812.

In considering these results one asks first whether they might not have been duplicated with normal blood. For it will be recalled that Landsteiner[8] was able to demonstrate autoagglutinins in the normal blood of several animal species, among them the rabbit. But, the blood of our recipients showed no clumping prior to the transfusions. Furthermore, Landsteiner's report shows that his agglutinins were vastly weaker than ours, being demonstrable at room temperature only when a large amount of serum was allowed to act on a few corpuscles. Nevertheless, we made attempts to demonstrate autoagglutinins in five normal rabbits by the same measures that had been employed with the transfused ones, but obtained consistently negative results.

Autofixation of Isoantibodies.

A remote possibility which suggested itself to account for the autoagglutination just described was the fixation on the donor cells and persistence throughout the washing process of isoantibodies present in the donor plasma and effective against the blood of the recipient. So unlikely did this seem that we had not considered it at the time of the above mentioned transfusions, but, secure in the realization that no foreign serum would reach the recipients, had omitted compatibility tests of them and the donors. Now an experiment was undertaken to determine whether normal isoagglutinins become fixed on the possessor's own cells when these latter are allowed to sediment out of citrated plasma in the cold. Bloods suitable for the work were obtained with some difficulty owing to the fact that isoantibodies in normal rabbits are weak and rather infrequent.[9]

Experiment 1.—By the examination of mixtures of whole citrated bloods five rabbits possessing isoagglutinins were selected. In mixtures of three parts of serum separated from the clot at 37°C. and one part of 5 per cent suspension in salt solution of the susceptible cells, definite clumping occurred in all cases at room temperature. Now portions of blood from the five animals were treated to the same slow, repeated washing by sedimentation in the ice box as in the case of the donors in the transfusion experiments with washed cells already described;

---

[9] Ottenberg, R., Kaliski, D. J., and Friedman, S. S., *J. Med. Research*, 1913–14, xxviii, 141.

and after the final washings, carried out with the help of the centrifuge, each cell sediment was tested as follows for serum agglutinins which might have become fixed on the corpuscles:

All possible supernatant fluid was drawn off; Locke's solution to about one-third the quantity of the original plasma was poured on; the cells were suspended for 10 minutes at 38–40°C.; and then they were thrown down by rapid, brief centrifugation. The salt solution was then immediately pipetted away and mixed in various proportions with 5 per cent suspensions of the cells that had originally been agglutinated by the plasma. As a control similar mixtures were made of cell suspensions and serum from the five animals, all kept from the time the original citrated specimens were drawn. The control serum had been removed from the clot after a few hours at incubator temperature and preserved in the ice box until needed.

In every case the salt solution clumped the cells acted upon by the control serum, while comparative tests in graded dilution showed that practically the entire original isoagglutinin content of the serum had passed into it.

The point disclosed by this experiment was most unexpected and of considerable theoretical interest. It is evident that normal isoagglutinins of the rabbit may become fixed upon the animal's own cells in the cold, to be given up again into salt solution at 37°C. Possibly in our transfusions with cells washed by sedimentation in the cold some isoagglutinins were thus carried over into the recipient. But this does not warrant the conclusion that an introduction of such isoagglutinins is the explanation of the clumping previously interpreted as autoagglutination. In the transfusions with whole citrated blood from compatible donors which resulted in what seemed to be autoagglutination the total amount of plasma injected into the recipient prior to the development of the clumping phenomenon was often considerably less and never very much more than the animal itself had to start with. Now granting that all of the alien plasma save the antibodies was removed from circulation, this would mean that the latter were left in about their original concentration in the donors, and at this concentration they had not been demonstrable *in vitro* when the donors were chosen. They cannot, then, have been responsible for the autoagglutination now seen in the recipient's shed blood and in suspensions of his preserved cells mixed with fresh serum. Nevertheless, transfusion experiments with cells washed in the warm were planned, but before they had been undertaken a new fact came to light. The corpuscles of normal rabbits having no demonstrable isoagglutinins in the plasma were themselves found to contain such antibodies in considerable amount.

Intracellular Agglutinins.

Klein[19] in 1902 claimed that extracts of red cells in salt solution or water showed agglutinative properties. His findings have met no general recognition, perhaps in part because of their conflict with the accepted rule that circulating antibodies are confined to the plasma, but also doubtless because of the irregularity of his results and the fact that the method used to obtain them is open to criticism. To obtain the agglutinins he ground the corpuscles with quartz sand; and salt solution ground with sand becomes endowed with the ability to cause a non-specific agglutination as we have repeatedly found. In the following experiment the cells were broken up by another means.

Experiment 2.—Three rabbits with plasmas that entirely failed to clump the cells of certain other rabbits in 9 to 1 and 1 to 9 mixtures of the whole bloods were bled 5 cc. each into 10 cc. of isotonic sodium citrate solution at 37°C. The corpuscles were at once packed with the centrifuge in a room maintained at body heat, and the supernatant fluid pipetted away as completely as possible, while still warm. The temperature of the room tended to go slightly above 38°C. because of the heating up of the centrifuge.

Now portions of each specimen of sedimented red cells were spread in thin layer on watch-glasses and dried in a current of air, a process requiring about 20 minutes. To the dried material of 0.7 to 1.0 cc. of cells, 1.0 to 1.7 cc. of doubly distilled water was added; and after the resulting thin paste had stood for a few minutes, it was centrifuged; the clear, deep red, supernatant fluid pipetted off; and to ten parts of this last one part of 9.0 per cent sodium chloride solution was added to restore approximate isotonicity. The fluid remained clear. It was now used in agglutination tests with corpuscles of the sort that had remained unagglutinated by the plasmas of the animals from which the cells for drying had come. Nine parts of the extract were used to one of a 50 per cent suspension of corpuscles in salt solution. In every case there was an almost immediate clumping of the cells into coarse floccull. Under the microscope the clumping was found to be a characteristic agglutination, with a candy-like stringing out of the adherent corpuscles when pressure was made on the cover-glass, and a resumption of the original form when pressure was relieved. There was no crenation of the cells. The floccull failed to break up when salt solution was added in quantity at room temperature, but fell apart immediately into the component cells when warmed to 37°C. in salt solution or the original cell extract; and in the latter at once reformed on cooling to or below 34.5°C.

---

[19] Klein, A., *Wien. klin. Woch.*, 1902, xv, 413.

A closer study of the intracellular agglutinins of rabbits such as were here found should have considerable interest. We have examined the corpuscles of eight human bloods for similar principles, according to the method described, but the findings were negative.

DISCUSSION.

With the demonstration that rabbit red cells contain a principle capable of agglutinating characteristically the corpuscles of other rabbits, our study of the autoagglutination induced by transfusion came to an end. In brief, we had found that the remarkable clumping of the cells in the shed blood of repeatedly transfused rabbits is due in most instances to the action of isoagglutinins developing in the recipient and effective upon the alien elements circulating amongst its own cells. Interagglutination of the donor cells by the plasmas introduced with them may sometimes play a subsidiary part in the phenomenon, as may possibly agglutination of the recipient's corpuscles by weak antibodies appearing in the donor's plasma during the course of the experiment. There remain instances in which, with all such causes of clumping ruled out, the recipient's corpuscles, taken prior to transfusion and preserved *in vitro*, are agglutinated by the animal's own serum obtained after several injections of alien blood. That these are genuine instances of induced autoagglutination remains uncertain because the possibility was not excluded that intracellular agglutinins derived from the donors underwent liberation in the recipient. The best evidence for a true autoagglutination lies in the not infrequent long persistence of the antibodies in the recipient's blood. In one such instance the clumping phenomenon remained well marked for at least 133 days after the last transfusion. During this period the plethora consequent on the introduction of the blood had been quickly succeeded by a profound anemia during which the hemoglobin fell to 27 per cent (Palmer), and this in turn was followed by a rapid recovery to normal and many weeks of good health.[3] Here the destruction and replacement of the circulating elements gave assurance that whatever the original source of the agglutinin, it was directed against the rabbit's own cells. An antibody of foreign origin would scarcely have remained so long in the blood stream. Tests with serum taken from other recipients when the clumping phenomenon was at its height, and preserved in the ice box, reinforce such findings. Thus, for example, it was observed that cells obtained from a recipient 86 days after the last transfusion, with an intervening plethora-anemia-recovery sequence, were agglutinated by serum from the animal preserved all this while. There are several facts which speak for the view that true autoagglutinins were induced in the transfused rabbits as a by-product to isoagglutinins. Our experiment in which normal isoagglutinins became fixed in the cold on their possessor's own cells shows how close is the antigenic relationship between the erythrocytes of different rabbits. And Ottenberg and Thalheimer[11] have made the significant observation with regard to the normal autoagglutinins of cats that: "Those strongly agglutinative sera which affect the red cells of a large number of other animals are usually those which are auto-agglutinative." The fact that the normal isoantibodies of cats are weak at most, while isohemolysins are entirely absent from the plasma would seem to indicate that, as in rabbits, the antigenic differentiation of the red cells is but slight. Numerous recent authors have noted that immunization with a single antigen may cause the development of antibodies of rather wide application. One need only suppose for the present case that this widening of application is such that the antibodies find a mark in the animal's own cells, as was Ehrlich's original conception.

The clumping observed in the shed blood of transfused rabbits never fails to bring together practically all of the cells. Yet our analysis of the content of the sera has shown in a number of instances that agglutinins were present only for certain cells in the circulating mixture. The apparent contradiction here seen is explained by the distribution of the susceptible cells within rouleaux. These latter, which fail to break up save when agglutination is strong, are brought together with the cells. If they have been broken up by washing with warm salt solution, and the cells dispersed, the agglutination becomes selective. Whenever the clumping of the shed blood is strong enough to disorganize the rouleaux and mass all the cells confusedly, agglutinins for all are regularly found to be present on serum analysis.

[11] Ottenberg, R., and Thalheimer, W., *J. Med. Research*, 1915–16, xxxiii, 213.

The fact that repeated subcutaneous or intraperitoneal injections with a compatible blood will cause the development of antibodies effective against such blood has been generally accepted since Ehrlich's study of induced isolysins in goats; but it has failed of recognition in its important practical bearing on the outcome of repeated transfusions in man. Our experience with rabbits repeatedly injected from compatible donors, especially such animals as showed a sudden and great blood destruction, serves to illustrate in little what must not infrequently occur in human beings. It follows that the most careful blood tests are called for with patients repeatedly retransfused at short intervals. In special, a thick spread of the patient's blood should be examined for autoagglutination which is often, as we have shown, an evidence that newly developed isoagglutinins are in circulation. Throughout the tests the temperature factor should be carefully controlled. Serum separated from the clot at 37°C. will often yield antibodies not demonstrable in that taken in the cold, and conversely agglutination mixtures examined at room temperature are far more likely to yield positive findings than those kept at blood heat.

Whether massive transfusions are preferable in pernicious anemia to repeated small ones remains uncertain. Sudden large increments of blood tend to lessen the reparative activity of the bone marrow.[4] But, on the other hand, they may also act to prevent fulminant destruction of the introduced blood by the recipient's serum antibodies. In the work already mentioned on experimental hemosiderosis in rabbits[2] some very large transfusions were given to animals that had developed strong serum antibodies, in the hope that abundant and continued blood destruction would ensue. This was never the case. The amount of antigen introduced so greatly exceeded that of the circulating antibodies that these latter failed to be destructive and plethora was maintained. It is a curious fact that in animals transfused 6 days out of every 7 during a period of 6 months the agglutinins gradually and completely disappeared, although following the first few injections they were often strong. A similar disappearance of precipitins upon long immunization has been recorded by Tschistowitsch and Nuttall.[12] Yet the transfused blood is somehow destroyed with great rapidity in animals frequently injected.

[12] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904, 127.

SUMMARY.

The massive agglutination observable in the shed blood of transfused rabbits, and associated not infrequently with sudden marked blood destruction, has a practical significance in connection with the untoward results of repeated transfusion from donors originally compatible; and it has special theoretical interest because the clumping of the cells is apparently an autoagglutination. To determine the actual source of the antibodies has been the object of the present work.

The agglutination in its most marked form has been traced to isoantibodies elicited by the presence in the body of corpuscles originally found compatible; and the frequently associated, rapid blood destruction is doubtless of similar origin. Occasionally antibodies develop in the donor bloods during the period of transfusion, but they are so weak as to be negligible. There remain instances of what would seem to be true autoagglutination due to serum bodies induced by the transfusions as a by-product, so to speak, in the manufacture of isoagglutinins. The antigenic relationship between the red cells of different rabbits is so close that normal isoagglutinins became fixed in the cold upon their elaborator's own corpuscles.

Agglutinins exist within the red cells of rabbits—as has been claimed by Klein. They are readily demonstrable in watery extracts of the dried corpuscles. Whether similar agglutinins ever exist within human cells remains to be determined. We have not found them in the normal corpuscles.

STUDIES ON THE PNEUMONIC EXUDATE.

V. THE RELATION OF PNEUMONIC LUNG PROTEASE ACTIVITY TO HYDROGEN ION CONCENTRATION, AND A CONSIDERATION OF THE ORIGIN OF THE ENZYME.

BY ROBERT N. NYE, M.D.

(*From the Research Laboratory of the Massachusetts General Hospital, Boston.*)

(Received for publication, August 26, 1921.)

That leucocytes contain an enzyme or enzymes capable of splitting native proteins to simpler nitrogenous compounds is an accepted fact at the present time. As early as 1877 Filehne[1] succeeded in obtaining from the sputum of a case of lung gangrene by extraction with glycerol an enzyme capable of dissolving fibrin and coagulated egg white in slightly alkaline media. A few years later Stolnikow[2] and Escherich[3] obtained similar results with extracts of sputa from cases of bronchitis, pneumonia, and phthisis. At that time the origin of the enzyme was disputed.

Müller[4] conducted a series of experiments relative to the action of empyema and abscess pus on fibrin and coagulated egg white, and found that the substances were readily dissolved. The sputa from cases of lobar pneumonia at the time of or just following crisis had a similar effect; whereas, before the crisis no enzyme action could be demonstrated. He also noted that pieces of pneumonic lung immersed in toluene water and placed in the incubator for several days became considerably lighter. He was of the opinion that the enzyme came from the destroyed white blood corpuscles. Simon,[5] following out Müller's views, studied chemically the autolysis of toluene water extracts of lungs from cases of lobar pneumonia. The extracts of five out of the six cases studied gave a primary acid reaction, and were subsequently neutralized or made slightly alkaline with calcium or sodium carbonate. After incubation for 5 to 8 days the average decrease in coagulable nitrogen, or increase in non-coagulable nitrogen, was 30 per cent; and an average of 30 per cent of the increase in non-coagulable nitrogen was accounted for by an actual increase in albumoses. The lung of the sixth case was in the stage of red hepatization; the extract was alkaline in reaction and underwent no autolysis. Control experiments on normal lung extracts showed no autolysis. About the same time Achalme[6] working with pus from various localities demonstrated the presence of a proteolytic enzyme capable of digesting fibrin and casein. He, also, concluded that this enzyme was derived from the leucocytes.

---

[1] Filehne, W., *Sitzungsber. physik.-med. Soc. Erlangen*, 1877, ix, 169, cited by Stolnikow.[2]

[2] Stolnikow, J., *St. Petersb. med. Woch.*, 1878, iii, 160.

[3] Escherich, T., *Deutsch. Arch. klin. Med.*, 1885, xxxvii, 196.

[4] Müller, F., *Verhandl. Cong. inn. Med.*, 1902, xx, 192.

[5] Simon, O., *Deutsch. Arch. klin. Med.*, 1901, lxx, 604.

More recently Opie[7] has carefully studied the proteolytic enzymes of sterile inflammatory exudates produced by intrapleural injections of an aleuronat suspension in rabbits and dogs. He concluded that there were two distinct proteolytic enzymes present in the exudates—a leucoprotease derived from the polymorphonuclear leucocytes and acting best in slightly alkaline media (0.2 per cent sodium bicarbonate), and a lymphoprotease derived from the mononuclear cells and acting best in slightly acid media (0.2 per cent acetic acid). The action of both enzymes was inhibited by the antiferment of normal beef serum. Jobling and Strouse[8] repeated some of Opie's work and came to essentially the same conclusions except that they believed there was also an erepsin-like enzyme present, active in both acid (0.2 per acetic acid) and alkaline (0.2 per cent sodium bicarbonate) media. Dernby[9] demonstrated in leucocytic suspensions enzymes capable of splitting peptone (optimum pH 7.6 to 8.0).

In a series of investigations on pneumonic exudates undertaken in this laboratory Lord[10] with cellular suspensions from pneumonic lungs ranging from pH 3.1 to 7.3 obtained visible erosion on Löffler's blood serum only at the higher end of the scale (pH 6.7 to 7.3). Peptone solutions were appreciably split by these cellular suspensions, and most complete splitting occurred at pH 5.2 or 6.3. He formulated the hypothesis that during the course of the disease in the pneumonic lung there is a gradual decrease in pH or increase in acidity. Primarily and at that time when the reaction of the exudate is about that of the circulating blood (pH 7.3 to 7.5) there is a digestion of the more highly organized proteins (fibrin, serum albumin, etc.) following the liberation of enzyme through cellular disintegration. With subsequent increase in acidity (beyond pH 6.7) the activity of this protease diminishes and conditions reach an optimum for the activity of the peptone-splitting enzyme. This peptonase or ereptase carries the splitting of the digestion products to amino-acids, absorbable as such; and resolution takes place.

It seemed advisable to determine quantitatively the digestive action of the exudate on one of the higher proteins and the relation of hydrogen ion concentration to the activity of the enzyme. As a substrate fibrin was chosen, since it is one of the higher proteins which must be broken down in the pneumonic lung during the process of resolution. Since a cellular suspension of the consolidated lung, rather than of the exudate alone, was used, it was necessary to rule out the action of enzymes contained in normal lung tissue.

---

[6] Achalme, *Compt. rend. Soc. biol.*, 1899, li, 568.

[7] Opie, E. L., *J. Exp. Med.*, 1906, viii, 410.

[8] Jobling, J. W., and Strouse, S., *J. Exp. Med.*, 1912, xvi, 269.

[9] Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179.

[10] Lord, F. T., *J. Exp. Med.*, 1919, xxx, 379.

EXPERIMENTAL.

1. Preparation of Substrate.—A mass of fresh horse fibrin about the size of a baseball was picked and washed practically free from adhering blood clot, and then passed through a meat chopper with a nut butter cutter attachment. The mash was centrifuged and washed repeatedly with water until the supernatant fluid was clear and colorless. The water was filtered off and the fibrin dried at 100°C. over night on a steam water bath. After grinding, the yield was 2.7 gm.

2. Preparation of Enzymes.—Portions of gray-red hepatization and normal lung from a case of Type I lobar pneumonia had each been previously ground in a meat chopper with a nut butter cutter attachment and kept in the ice box several weeks following the addition of chloroform and toluene. The consolidated lung mash was washed through two thicknesses of sterile gauze with 0.85 per cent sterile salt solution and the washings were centrifuged and washed four or five times with salt solution. The final centrifuging (No. 7 stop for 7 minutes) yielded 5 cc. of "cells," which were made up to 15 cc. with sterile salt solution. The normal lung mash was ground with sterile sand in a sterile mortar; and, after separating the sand from the suspension by centrifuging at low speed, treated as above. The yield was only 0.6 cc. of "cells;" these were made to 15 cc. with salt solution.

3. Method.—To dry 60 cc. sterile flasks were added phosphate mixtures, fibrin, 10 cc. of sterile water (Nos. 6 to 15 inclusive, 15 cc.), and 1.0 cc. of cellular suspensions (Nos. 6 to 15 inclusive, 1.5 cc.) (Table I). To those flasks intended to have pH values of 4.0 and 5.0 were added 4 drops of a 2 per cent aqueous solution of sodium alizarin sulfonate, and to the remainder 2 drops of a 0.01 per cent solution of phenol red. The flasks were adjusted approximately to the desired pH by the addition of normal and 0.1 normal hydrochloric acid and sodium hydroxide and comparison with a set of standard pH solutions prepared according to Clark and Lubs.[11] Water was added to make the total volume 20 cc. (Nos. 6 to 15 inclusive, 30 cc.). After thorough agitation 10 cc. samples were removed from Flasks 6 to 15 inclusive. 1 cc. of toluene was added to each flask and all were tightly corked and placed in the incubator at 37.5°C. for 5 days.

Cultures taken after 12 hours incubation and at the end of the experiment were sterile. During incubation there was no appreciable change in reaction.

The coagulable proteins were precipitated by the addition of trichloroacetic acid (3 per cent of total volume) according to Greenwald.[12] The tubes were

---

[11] Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1916, xxv, 479.

[12] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

TABLE I.

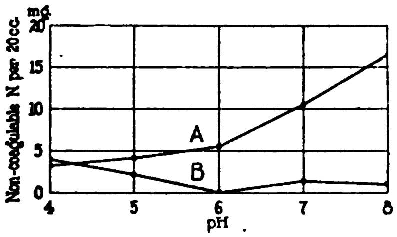
The Composition of the Various Digestion Mixtures and the Results of the Determinations.

Tube No.	Fibrin.	Pneumonic lung cellular suspension.	Normal lung cellular suspension.	0.5 M KH_2PO_4.	0.5 M $Na_2HPO_4 \cdot 2H_2O$.	Water, acid, and alkali.	pH	Non-coagulable nitrogen per 20 cc.			Amino nitrogen per 20 cc.		$\frac{\text{Amino nitrogen}}{\text{Non-coagulable nitrogen}} \times 100.$
								Before digestion.	After digestion.	Actual digestion.	After digestion.	Actual digestion.	
	gm.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1	0.30*			2.0		18.0	4.0	—	1.9	—	0.46	—	
2	0.30			1.8	0.2	18.0	5.0	—	2.5	—	0.50	—	
3	0.30			1.6	0.4	18.0	6.0	—	4.2	—	0.52	—	
4	0.30			0.4	1.6	18.0	7.0	—	5.4	—	0.62	—	
5	0.30				2.0	18.0	8.0	—	6.3	—	0.74	—	
6		1.5		3.0		25.5	4.0	1.0	1.2	0.2	0.24	—	
7		1.5		2.7	0.3	25.5	5.0	0.6	1.0	0.4	0.28	—	
8		1.5		2.4	0.6	25.5	6.0	0.8	1.7	0.9	0.34	—	
9		1.5		0.6	2.4	25.5	7.0	0.5	2.0	1.5	0.54	—	
10		1.5			3.0	25.5	8.0	0.7	2.5	1.8	0.56	—	
11			1.5	3.0		25.5	4.0	0.1	0.3	0.2	—	—	
12			1.5	2.7	0.3	25.5	5.0	0.1	Lost.	?	—	—	
13			1.5	2.4	0.6	25.5	6.0	0.2	0.8	0.6	—	—	
14			1.5	0.6	2.4	25.5	7.0	0.2	0.3	0.1	—	—	
15			1.5		3.0	25.5	8.0	0.2	0.6	0.4	—	—	
16	0.30	1.0		2.0		17.0	4.0	—	5.8	2.7	1.02	0.32	12
17	0.30	1.0		1.8	0.2	17.0	5.0	—	7.7	4.2	1.22	0.44	11
18	0.30	1.0		1.6	0.4	17.0	6.0	—	11.3	5.4	1.58	0.72	13
19	0.30	1.0		0.4	1.6	17.0	7.0	—	17.9	10.5	2.76	1.60	15
20	0.30	1.0			2.0	17.0	8.0	—	25.2	16.4	3.26	1.96	12
21	0.30		1.0	2.0		17.0	4.0	—	6.2	4.0	—	—	
22	0.30		1.0	1.8	0.2	17.0	5.0	—	4.6	1.6†	—	—	
23	0.30		1.0	1.6	0.4	17.0	6.0	—	5.0	0.0	—	—	
24	0.30		1.0	0.4	1.6	17.0	7.0	—	7.1	1.4	—	—	
25	0.30		1.0		2.0	17.0	8.0	—	7.8	0.9	—	—	

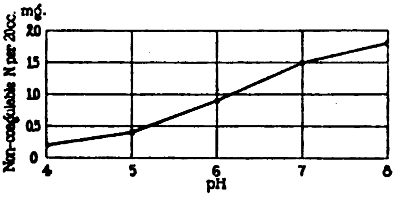
* Total nitrogen determination 0.042 gm.

† Estimating No. 12 flask after digestion as 0.5 mg.

stoppered, shaken, and allowed to stand 12 to 15 hours in the ice box before filtering. Nitrogen determinations on the filtrates were made by Folin's[13] macro Kjeldahl method, using 5 cc. samples and 5 cc. of his digestion mixture. Duplicate determinations were made on all filtrates. The non-coagulable nitrogen representing the actual digestion of the fibrin by the cellular enzymes was obtained by subtracting the sum of the non-coagulable nitrogen of the fibrin and lung controls from that of the digestion mixture. The results are expressed as the average amount of non-coagulable nitrogen present in 20 cc. Amino nitrogen was determined by Van Slyke's[14] nitrous acid method using 2 cc. samples of the filtrates following the precipitation of the coagulable nitrogen. Blank determinations made with distilled water and with distilled water saturated with toluene eliminated the presence of traces of the preservative as a source of error. Results are expressed as the amount of amino-acid nitrogen present in 20 cc.



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Proteolytic activity of pneumonic (*A*) and normal (*B*) lung cellular suspensions at varying hydrogen ion concentrations.

TEXT-FIG. 2. Autolysis of pneumonic lung cellular suspensions at varying hydrogen ion concentrations.

The results clearly show (Text-fig. 1) that there is present in the pneumonic lung an enzyme, or enzymes, capable of digesting horse fibrin. Similar results were obtained with cellular suspensions from the consolidated lungs of lobar pneumonias other than those caused by Type I pneumococcus. In the range of reactions chosen as physiological possibilities the enzyme shows the greatest activity at the most alkaline end of the scale (pH 8.0). At this hydrogen ion concentration it was able to convert 46 per cent[15] of the fibrin originally present to a non-coagulable form after 5 days incubation. The digestion at pH 4.0 is comparatively slight; and there is a gradual increase of digestion corresponding to decrease in acidity. Appreciable autolysis of the pneumonic lung controls occurred, slight at pH 4.0 and 5.0 with a gradual increase towards the alkaline side (Text-fig. 2). Presumably the same enzyme is responsible for both digestive processes. The amino-acid nitrogen determinations show that in the pH 7.0 digestion mixture the protein splitting was most complete. This corresponds roughly to the optimum hydrogen ion concentration of the pneumonic lung peptonases or ereptases, as reported by Lord[10] and confirmed in a paper to follow. The cellular suspension controls of normal lung show an appreciable digestion of the fibrin at pH 4.0, and practically no increase in non-coagulable nitrogen in the less acid media (Text-fig. 1).

---

[13] Folin, O., and Wright, L. E., *J. Biol. Chem.*, 1919, xxxviii, 461.

[14] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.

[15] Calculated from the following figures (Table I): total nitrogen of fibrin, 42 mg.; non-coagulable nitrogen of fibrin at pH 8.0, 6.3 mg.; and actual increase in non-coagulable nitrogen in digestion mixture at pH 8.0, 16.4 mg.

DISCUSSION.

This protease can be conceived as arising from one or more of four sources: (*a*) the pneumococci, (*b*) the cells present in the normal lung, (*c*) the blood serum, and (*d*) the cells of the pneumonic exudate.

Avery and Cullen[16] have been able to obtain from pneumococci intracellular enzymes capable of digesting fibrin. With fibrin freshly prepared from rabbit blood and the bile extract from the organisms contained in 200 cc. of a plain bouillon culture of Pneumococcus Type II, the total actual digestion at pH 7.4 amounted to an increase of 0.26 mg. of amino nitrogen. With 0.2 gm. of commercial fibrin and a similar amount of enzyme, their actual increase in amino nitrogen was only 0.11 mg. Furthermore, since their enzyme contained non-coagulable nitrogen and in addition underwent autolysis on incubation, it is conceivable that in their digestion mixtures a portion of the increase in amino nitrogen might have been due not to an enzyme digesting native protein but to one autolyzing the enzymatic non-coagulable nitrogen—a peptonase, the presence of which they clearly demonstrated in extracts of the pneumococcus. With 0.3 gm. of fibrin and 0.33 cc. of washed cellular suspension from the pneumonic lung the total increase in amino nitrogen at pH 7.4 fell somewhere between 1.60 mg. (pH 7.0) and 1.97 mg. (pH 8.0). Stained smears of the cellular suspension showed no intact organisms. In the washing of the cellular material in preparation one would expect the enzyme liberated from the disintegrated pneumococci to have been lost. Undoubtedly the pneumococcus protease contained in 0.33 cc. of washed cellular suspension is negligible when considering digestion amounting to 1.60 to 1.97 mg. of amino-acid nitrogen.

---

[16] Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1920, xxxii, 547.

Opie[7] has shown that sterile pleural exudates containing an excess of mononuclear cells exhibit maximum proteolytic activity in slightly acid media (0.2 per cent acetic acid). The phagocytic mononuclear or endothelial cells are derived from the endothelial cells lining the blood and lymph vessels.[17] A cellular suspension obtained from a normal lung, or to a less degree from the true lung substance of a pneumonic lung, would contain, so far as those cells which are known to contain proteolytic enzymes are concerned, chiefly endothelial cells. Consequently from Opie's work one would expect maximum digestion in a slightly acid medium. This was exactly what happened in the normal lung cellular suspension and fibrin digestion mixtures. Since nearly twice as much fibrin was digested at pH 4.0 by the 0.04 cc. of normal lung cellular suspension as by the 0.33 cc. of pneumonic cellular suspension, it can be safely assumed that the latter did not contain as much true lung substance as the former. In spite of the presence of at least an equal amount of true lung substance, the normal lung suspension as compared with that of the pneumonic lung showed but slight digestion in the slightly acid, neutral, and slightly alkaline digestion mixtures. Hence, the enzyme, or enzymes, derived from the true lung substance of a pneumonic lung play but a slight rôle in the proteolysis of fibrin by the pneumonic lung cellular suspension except in moderately acid media (pH 4.0).

Proteases or trypsin-like ferments are known to be normally present in blood serum; but they are held in abeyance by the presence of antitrypsin. Delezenne and Pozerski[18] observed that the antitryptic power of the serum is lost following extraction with chloroform. It is conceivable that in lung cellular suspensions preserved with chloroform and toluene the serum proteases become potentially active. However, frequent washings in preparation of the enzyme previous to the experiment would certainly remove them.

---

[17] Mallory, F. B., The principles of pathologic histology, Philadelphia and London, 1918.

[18] Delezenne, C., and Pozerski, E., *Compt. rend. Soc. biol.*, 1903, lv, 327, 690.

The protease of pneumonic lung cellular suspensions is derived chiefly from the leucocytes of the exudate. The other possible sources play but a minor rôle. Confirming the work of many other investigators, it was found that this enzyme is most active in a slightly alkaline medium (pH 8.0).

SUMMARY.

1. Washed cellular suspensions of pneumonic lungs, previously preserved with chloroform and toluene, contain a protease or proteolytic ferment, derived chiefly from the leucocytes of the exudate.

2. This protease is able to convert horse fibrin to a non-coagulable split product. In a pH range of 4.0 to 8.0 the digestion is slight at the most acid end. With decrease in acidity there is a gradual rise in activity to pH 6.0, and then a sharp increase up to the maximum digestion at pH 8.0. Judging from the amino nitrogen determinations, the most complete splitting occurs at pH 7.0. The degree of autolysis occurring in the pneumonic lung cellular suspension controls suggests that the active enzyme is identical with that causing the digestion of the fibrin.

3. Washed cellular suspensions of normal lungs, previously preserved with chloroform and toluene, contain a proteolytic enzyme which is active in a moderately acid medium (pH 4.0) and essentially inactive in less acid, neutral, and slightly alkaline media.

The author is indebted to Dr. F. T. Lord for many helpful suggestions.

THE TRANSMISSION OF AGGLUTININS OF BACILLUS ABORTUS FROM COW TO CALF IN THE COLOSTRUM.

BY RALPH B. LITTLE, V.M.D., AND MARION L. ORCUTT.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, September 2, 1921.)

In the work on infectious abortion associated with *Bacillus abortus* carried on in this Department since 1917,[1,2] the blood serum and transudates of fetuses have been tested from time to time to determine the relation between the concentration of agglutinins in the blood serum of mother and fetus. The results, given in Table I, indicate that even when the serum of the mother has a high agglutinin content,

TABLE I.

Agglutinin Content of Blood of Dam and Aborted Fetus.

Case No.	Blood serum.		*B. abortus* isolated.*	Remarks.
	Dam.	Fetus.		
493	1:1,280	1:40	Yes.	
374	1:1,280	1:80	"	1:20 to 1:80 show the same degrees of agglutination. Abdominal and thoracic transudates negative.
652	1:1,280	0	"	
506	?	0	"	
270	1:1,280	0	"	
590	1:320	1:40	"	
595	1:320	1:20	"	
579	1:80	1:20	No.	
509	?	1:40	Yes.	
518	1:1,280	1:20	"	
668	1:320	1:20	"	Colostrom 1:2,560.
671	1:1,280	1:20	"	" 1:2,560.

* From autopsy records of the Department.

---

[1] Smith, Theobald, *J. Exp. Med.*, 1919, xxx, 325.

[2] Smillie, E. W., Little, R. B., and Florence, L., *J. Exp. Med.*, 1919, xxx 341

little or none is found in the fetal blood. The agglutinin figures given represent the agglutination limits of series of dilutions.

Tests of the blood of calves within the first few days of life presented different results. In some a relatively high titer was observed. The variation from case to case was finally explained by the absorption of agglutinins in the colostrum, as the experiments reported below amply demonstrate.

The literature on the transmission of various antibodies in the milk is fairly rich. Only a few publications bear more directly on the subject in hand. For a thorough review up to 1912 the reader is referred to Famulener's article.[3] In his own investigations on this subject, Famulener immunized goats with sheep blood corpuscles. He tested the antibody content of the mother's serum and colostrum at time of parturition and the blood of the kids before and after they had ingested colostrum. He found that in immunized goats the colostrum was rich in antibodies but in the kids no antibodies were present until after taking the colostrum. He observed that the titer of the colostrum in many cases was above that of the mother's serum, and that the milk quickly dropped in antibody content. From his experiments he concluded that the hemolytic antibodies in the young are received through the colostrum and absorbed by the young from the gastrointestinal tract, but that this absorption can only take place during the first few days after birth.

Rettger and White[4] in 1918 and Rettger[5] in 1920 made the statement that, without exception, cows with a positive agglutination to *B. abortus* gave birth to calves which showed the same reaction. This statement is true only after the first feeding with colostrum.

Recently Reymann[6] in studying the transfer of normal agglutinins from mother to young also worked with goats, but he brings out no new points concerning the transmission of agglutinins. Strangely enough he fails to refer to Famulener's results.

EXPERIMENTAL WORK.

The experiments to be reported differ from those of others in that they deal with the naturally acquired and stored agglutinins as the result of a definite infectious disease of cows, localized in the fetal membranes. Earlier experiments deal only with agglutinins experimentally induced.

---

[3] Famulener, L. W., *J. Infect. Dis.*, 1912, x, 332.

[4] Rettger, L. F., and White, G. C., *Storrs Agric. Exp. Station, Bull. 93*, 1918, 225.

[5] Rettger, L. F., *Abstr. Bact.*, 1920, iv, 13.

[6] Reymann, G. C., *J. Immunol.*, 1920, v, 227.

The procedure for the collection and dilution of the blood serum for the agglutination tests is the same as described in a previous paper.[2] The milk was handled as follows:

The samples were centrifuged and the cream layer was removed. This was necessary in order to obtain a clear whey; however, with some samples of colostrum which were very thick the centrifuging was omitted. The samples were then warmed to about 35°C. and several drops (8 to 10) of a solution of a rennet tablet in sterile distilled water were added. The tubes were well shaken and left standing in the water bath 15 to 20 minutes. By that time a firm coagulum had formed. In order to separate the whey quickly, the tubes were then put into warmer water, at about 45°C. This caused the coagulum to shrink and a clear whey to separate. This was drawn off and used for the agglutination tests. Frequent tests have shown that the rennet does not influence the agglutinins present.

The preparation and standardization of the antigen for blood and milk was as follows:

Bacillus abortus was grown on agar at 37°C. for 24 to 48 hours. The growth was washed off in 0.85 per cent salt solution. This was standardized to a given opacity by means of the Gates instrument.[7] A depth of disappearance of 2.4 cm. was taken as the standard. Each lot of antigen was made up to this density before use.

In Table II the agglutination titers of the blood and colostrum of seven cows and their calves soon after birth are given to illustrate the conditions usually found. Of these offspring, three (Nos. 488, 619, and 484A) were tested after suckling and agglutinins found in their blood. Two were negative when tested before suckling (Nos. 484B and 625). Of two premature calves, one had agglutinins in the blood (No. 567), the other not (No. 483).

These random illustrations showed the need of determining by suitably controlled tests the relation of the ingestion of colostrum to the agglutinin titer. In Table III illustrations of such tests are given. In the first case agglutinins appeared in the blood of the calf after it had suckled a cow (not its mother) whose colostrum had a high titer. In the second, the calf was fed colostrum artificially and agglutinins appeared but of a lower titer than in the preceding calf

[7] Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

TABLE II.

Material tested.	Date of blood test.	Dilutions.										Control.
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	
Cow 484 and twin calves A and B, born Mar. 23, 1921. A had nursed before the first bleeding; B, much weaker, had not nursed until after the first bleeding.												
	1921											
Cow's blood	Mar. 23	C.*	C.	++++	+	–	–	–				–
Colostrum	" 23	"	++++	++++	++	+	–	–				–
Blood, Calf A	" 23	+++	+	+	–	–	–	–				–
" " "	" 24	C.	+++	+	+	–	–	–				–
" " B	" 23	–	–	–	–	–	–	–				–
" " "	" 24	C.	++	+	–	–	–	–				–
Cow 488 and calf which had nursed before blood test. Calf born Feb. 18, 1921.												
Cow's blood	Feb. 18	C.	C.	C.	++++	+++	++	±	–	–	–	–
Colostrum	" 18	++++	++++	++++	++++	+++	C.	C.	C.	C.	++	–
Calf's blood	" 18	++++	+++	++	++	++++	"	"	+++	±	–	–
" "	" 19	C.	+++	+	++	C.	"	"	++++	+	–	–
Cow 619 and calf which had nursed before first bleeding. Calf born June 9, 1921.												
Cow's blood	June 9	C.	C.	C.	C.	C.	C.	C.				–
Colostrum	" 9	"	"	"	"	"	"	++++	+++	++	++	–
Calf's blood	" 9	"	++++	++++	++++	++++	++++	++++				–
" "	" 10	++++	++++	C.	C.	C.	C.	C.				–

Cow 625 and calf which had not nursed before first bleeding. Calf born July 3, 1921.												
Cow's blood............	July 5	C.	C.	C.	C.	++	++	++				–
Calf's "	" 3	–	–	–	–	–	–	–				–
" "	" 4	C.	C.	++++	++	+	±					
Cow 483 and 8 mo. calf (premature) which had not nursed before first bleeding. Calf born Mar. 5, 1921.												
Cow's blood............	Mar. 5	C.	C.	C.	C.	++++	++++	+++				–
Colostrum.............	" 5	++++	++++	++++	++++	++++	++++	+++	++			–
Calf's blood............	" 5	–	–	–	–	–	–	–				–
" "	" 6	+++	+	–	–	–	–	–				–
" "	" 7	++++	+	–	–	–	–	–				–
Cow 477 and calf which may have nursed before bleeding.												
Cow's blood............	May 28	C.	C.	C.	++	+	–	–				–
Colostrum.............	" 28	++++	++++	+++	++	+	–	–				–
Calf's blood............	" 28	+	–	–	–	–	–	–				–
" "	" 31	++++	+	–	–	–	–	–				–
Cow 567 and premature unfed calf. *B. abortus* found in the organs subsequently.												
Cow's blood............	Feb. 16	C.	C.	C.	C.	+++	++	–				–
Colostrum.............	" 16	+++	++++	++++	++++	+++	+	–				–
Blood of fetus..........	" 16	++++	++++	+++	++	+	–	–				–

---

* The plus signs stand for degrees of clumping from almost complete (+ + + +) to just visible clumping (+). The minus sign means less than 1:20, and no clumping in controls. The letter C stands for complete clearing of the fluid.

TABLE III.

<table>
<tr><th rowspan="2">No. of dam.</th><th colspan="2">Titer.</th><th rowspan="2">Age of calf when blood was tested.</th><th rowspan="2">Titer of blood.</th><th rowspan="2">Remarks.</th></tr>
<tr><th>Blood.</th><th>Colostrum.</th></tr>
<tr><td colspan="6">Calf of Cow 627, born July 7, 1921, at 9.40 a.m. Restrained from taking colostrum until after three examinations of the blood, then allowed to suckle a high titer cow (No. 626) which had given birth to a calf the same day.</td></tr>
<tr><td>627</td><td>1:160</td><td>1:320</td><td>1 hr., 50 min.</td><td>–</td><td></td></tr>
<tr><td></td><td></td><td></td><td>7 hrs., 10 "</td><td>–</td><td>Calf received 1 quart of milk free from agglutinins.</td></tr>
<tr><td></td><td></td><td></td><td>21 " 30 "</td><td>–</td><td></td></tr>
<tr><td></td><td colspan="4">Calf placed with Cow 626.</td><td></td></tr>
<tr><td>626</td><td>1:1,280</td><td>1:2,560</td><td>32 hrs.</td><td>1:640</td><td></td></tr>
<tr><td></td><td></td><td></td><td>46 " 50 min.</td><td>1:640</td><td></td></tr>
<tr><td colspan="6">Calf born of a high titer cow (No. 683), July 21, 1921, at 12.30 p.m. Restrained from taking colostrum until after two examinations of blood, then fed dam's colostrum which had been refrigerated and warmed.</td></tr>
<tr><td>683</td><td>1:1,280</td><td>1:2,560</td><td>4 hrs., 45 min.</td><td>–</td><td>Calf received 42 ounces of milk free from agglutinins.</td></tr>
<tr><td></td><td></td><td></td><td>21 " 10 "</td><td>–</td><td></td></tr>
<tr><td></td><td colspan="4">Calf received 36 ounces of colostrum.</td><td></td></tr>
<tr><td></td><td></td><td></td><td>28 hrs., 30 min.</td><td>1:40</td><td>Calf received 92 ounces of colostrum.</td></tr>
<tr><td></td><td></td><td></td><td>45 " 10 "</td><td>1:160</td><td></td></tr>
<tr><td colspan="6">Calf born of a high titer cow (No. 634), July 12, 1921, at 11 a.m. Restrained from taking colostrum until after three blood examinations, then fed dam's colostrum which had been refrigerated and warmed.</td></tr>
<tr><td>634</td><td>1:320</td><td>1:640</td><td>1 hr.</td><td>–</td><td></td></tr>
<tr><td></td><td></td><td></td><td>6 hrs.</td><td>–</td><td>Calf received 1 quart of milk free from agglutinins.</td></tr>
<tr><td></td><td></td><td></td><td>22 " 30 min.</td><td>–</td><td></td></tr>
<tr><td></td><td colspan="4">Calf received 33 ounces of colostrum.</td><td></td></tr>
<tr><td></td><td></td><td></td><td>29 hrs.</td><td>–</td><td></td></tr>
<tr><td></td><td></td><td></td><td>45 "</td><td>–</td><td></td></tr>
<tr><td colspan="6">Calf born of a high titer cow (No. 657), Sept. 16, 1921, at 4.20 p.m. Restrained from taking colostrum or milk until after two examinations of the blood, then fed milk free from agglutinins.</td></tr>
<tr><td>657</td><td>1:1,280</td><td>1:1,280</td><td>40 min.</td><td>–</td><td></td></tr>
<tr><td></td><td></td><td></td><td>16 hrs., 40 "</td><td>–</td><td></td></tr>
<tr><td></td><td colspan="4">Calf received milk.</td><td></td></tr>
<tr><td></td><td></td><td></td><td>2 days, 16 hrs., 40 min.</td><td>–</td><td></td></tr>
<tr><td></td><td></td><td></td><td>5 " 16 " 40 "</td><td>–</td><td></td></tr>
</table>

TABLE III—*Concluded.*

No. of dam.	Titer.		Age of calf when blood was tested.	Titer of blood.	Remarks.
	Blood.	Colostrum.			
Calf born of a low titer cow (No. 653), July 3, 1921, at 2 p.m. Restrained from taking colostrum until after one examination of the blood, then allowed to suckle a high titer cow (No. 654) which had given birth to a calf the same day. Milk was collected from Cow 653, 4 days after parturition, and blood 25 days after.					
653	1:20	1:160	1 hr., 35 min.	–	
	1:20	–(milk)			
		Calf placed with Cow 654.			
654	1:320	1:1,280	18 hrs., 50 min.	1:320	
Calf born of a high titer cow (No. 655), July 23, 1921, at 5.30 a.m. Restrained from suckling until after one blood examination, then allowed to suckle dam.					
655	1:640	1:1,280	5 hrs., 5 min.	–	
		Calf placed with Cow 655.			
			11 hrs., 20 min.	1:160	
			32 " 10 "	1:640	
Calf born of a high titer cow (No. 684), July 22, 1921, at 11.30 a.m. Restrained from suckling until after two blood examinations, then allowed to suckle dam.					
684	1:640	1:1,280	2 hrs., 15 min.	–	
			6 " 10 "	–	
		Calf placed with Cow 684.			
			11 hrs., 50 min.	1:160	
			23 " 15 "	1:320	

which suckled naturally. In the third illustration, the calf although fed colostrum failed to show agglutinins in its blood. In the fourth case, the colostrum of the mother had a high titer but the calf's blood was negative and remained so for nearly 6 days while the colostrum was being withheld. Later tests were interfered with by the death of the calf. The remaining cases reenforce the data given in the others. In the seventh, the agglutinins in the blood of the calf rose quite rapidly from the 11th to the 23rd hour.

In Table IV, the results of agglutination tests on the blood serum and colostrum of the mother and the blood serum of the new-born calf before and after the first meal of colostrum are presented in more detail to add to the data already presented and to show certain slight

TABLE IV.

No. of dam.	Age of calf when blood was tested.	Material tested.	Dilutions.								Control.
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	
Calf born of a high titer cow (No. 655), at 5.30 a.m. Restrained from suckling until after one blood examination, then allowed to suckle the dam.											
655		Cow's blood.	C.	C.	C.	++++	+++	+	–		–
655		Colostrum.	"	"	"	C.	++++	++	+		–
Calf.	5 hrs., 5 min.	Calf's blood.	–	–	–	–	–	–	–		–
		Calf allowed to suckle dam.									
"	11 " 20 "	Calf's blood.	C.	++++	++	+	–	–	–		–
"	32 " 10 "	" "	"	C.	++++	+++	+	±	–		–
Calf born of a low titer cow (No. 664), at 5.20 p.m. Restrained from suckling until after one blood examination, then allowed to suckle the dam.											
664		Cow's blood.	C.	++	+	±	–	–	–		–
664		Colostrum.	+++	+++	++	+	–	–	–		–
Calf.	20 min.	Calf's blood.	–	–	–	–	–	–	–		–
		Calf allowed to suckle dam.									
"	4 hrs.	Calf's blood.	++	+	+	–	–	–	–		–
"	6 " 10 min.	" "	++++	+++	++	+	–	–	–		–
"	14 " 30 "	" "	++++	++++	+++	++	+	–	–		–

Calf born of a high titer cow (No. 669), at 1.20 p.m. Restrained from suckling until after one blood examination, then allowed to suckle the dam.

669		Cow's blood.	C.	C.	C.	++++	+	−	−	−	−
669		Colostrum.	+++	++++	++++	++++	+++	+++	++	+	−
Calf.	35 min.	Calf's blood.	−	−	−	−	−	−	−	−	−
		Calf allowed to suckle dam.									
"	2 hrs., 40 "	Calf's blood.	+	++	+++	+	+	−	−	−	−
"	5 " 40 "	" "	++++	+++	+++	++++	+++	++	+	−	−
"	19 " 40 "	" "	++++	++++	+++	++	+++	+++	+++	++	−
"	67 " 40 "	" "	C.	C.	++++	++++	+++	++	±	−	−

Calf born of a high titer cow (No. 411), at 3.30 p.m. Restrained from suckling until after one blood examination, then allowed to suckle the dam.

411		Cow's blood.	C.	C.	C.	++++	+++	+	−	−	−
411		Colostrum.	+++	+++	++++	++++	+++	++++	++++	++++	−
Calf.	35 min.	Calf's blood.	−	−	−	−	−	−	−	−	−
		Calf allowed to suckle dam.									
"	1 hr., 50 "	Calf's blood.	++	+	−	−	−	−	−	−	−
"	3 hrs., 50 "	" "	C.	++++	++++	++++	+++	++	−	−	−
"	18 "	" "	"	++++	++++	+++	+++	+++	++	+	−
"	72 "	" "	"	++++	++++	++++	++++	+++	+	−	−

irregularities in the flocculation of *Bacillus abortus* in the series of tubes containing dilutions of the colostrum and of the blood serum of the calf. These irregularities are evident in the calf's blood serum and colostrum of No. 669 and in the colostrum of No. 411. Similar irregularities are shown in Table II (Nos. 488 and 619).

In three cases of Table IV, the agglutinin titers of blood serum and colostrum of the dam were high but that of the calf's serum was negative until after the first meal. The fourth case (No. 664) presents a lower titer but the transmission of agglutinins to the calf is nevertheless in evidence. The higher concentrations of agglutinins in the colostrum than in the blood serum of the mother is indicated in three out of the four cases. In two cases the titer of the calf's serum rose above that of the mother.

DISCUSSION.

The tabulated observations and experiments are sufficient to establish the fact that even when the blood and colostrum of the dam have a relatively high content of agglutinins towards *Bacillus abortus*, the blood of the new-born calf, with rare exceptions, is free from such antibodies until the animal has suckled and taken in the colostrum. The only exception found is the last case in Table II. In this premature calf the autopsy record shows that the stomachs were still free from food and that *Bacillus abortus* was present in the spleen, liver, and kidneys. The antibodies which appear in the calf's blood are absorbed from the digestive tract. The precise age of the calf after which agglutinins fail to enter the blood as such has not been determined. Experiments bearing on this aspect of the subject are under way.

When colostrum was withheld and milk of a low or negative agglutinin titer substituted, agglutinins failed to appear or accumulate in the blood of the calf. The longest period following birth during which the colostrum was withheld in the various tests was 5 days.

A study of the tables shows, what has been pointed out by others, that the concentration of the agglutinins in the colostrum may exceed that of the blood at and immediately after parturition.

The rate of absorption into the blood is shown to be fairly rapid. Agglutinins began to appear slightly over an hour after the calf had ingested colostrum. They were nearly at the maximum concentration 5 hours after feeding.

The test upon all samples was carried out as shown in detail in Table IV. The dilutions were made in series from 1:20 to 1:1,280 or 1:2,560, each tube being one-half the dilution of the preceding tube of the series. By this method certain irregularities in the agglutinin concentration in the colostrum were occasionally encountered. In certain tubes of a series the flocculation was stronger than in a higher, preceding concentration. In such cases the irregularities may appear in the blood serum of the calf after taking colostrum, but the blood serum of the mother does not present them.

CONCLUSIONS.

The agglutinins towards *Bacillus abortus* found in the blood serum of new-born calves are obtained from the mother through the colostrum. Calves at birth, unfed, are without agglutinins. The problem of the production of agglutinins by the fetus in whose tissues *Bacillus abortus* has multiplied and which is subsequently expelled prematurely is not touched by these observations.

THE ERYTHROPOIETIC ACTION OF GERMANIUM DIOXIDE.

By FREDERICK S. HAMMETT, Ph.D., JOSEPH E. NOWREY, Jr., and JOHN H. MÜLLER, Ph.D.

(*From The Wistar Institute of Anatomy and Biology, and The John Harrison Laboratory of Chemistry of the University of Pennsylvania, Philadelphia.*)

(Received for publication, September 20, 1921.)

Not only have the substances which by their commonness, such as the salts of sodium, potassium, and calcium, or by their striking and unexpected action on living tissues, such as radium and its derivatives, been intensively studied, but also many observations have been reported of the therapeutic trials and physiological action of the rarer elements. Cerium,[1] cesium,[2] erbium,[3] indium,[4] lanthanum,[5] neodmiumy,[5] palladium,[6] praseodymium,[1] rhodium,[7] rubidium,[8] ruthenium,[9] samarium,[10] selenium,[11] tellurium,[12] thallium,[13] thorium,[14] titanium,[15] vanadium,[16] yttrium,[17] and zirconium[18] have all received some attention from the physiological point of view.

---

[1] Frouin, A., *Compt. rend. Acad.*, 1920, clxx, 1471.

[2] Kaiser, L., *Arch. néer. physiol.*, 1918–19, iii, 587.

[3] Mines, G. R., *J. Physiol.*, 1910, xl, p. xlviii.

[4] Gerber, G., *Compt. rend. Soc. biol.*, 1910, lxix, pt. 2, 104.

[5] Rénon, L., *Bull. et mém. Soc. méd. hôp. Paris*, 1920–21, xliv, 602.

[6] Ascoli, M., and Izar, G., *Biochem. Z.*, 1907, vi, 192.

[7] Rémond, A., *Schweiz. Apoth. Z.*, 1919, lvii, 242.

[8] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 567.

[9] Kalle and Co., German Patent No. 289,620, July 31, 1913.

[10] Esnault and Bron, *Bull. et mém. Soc. méd. hôp. Paris*, 1920–21, xliv, 606.

[11] Duhamel, deB.-G., *Compt. rend. Soc. biol.*, 1919, lxxxii, 724.

[12] Shie, M. D., and Deeds, F. E., *Pub. Health Rep., U. S. P. H.*, 1920, xxxv, 939.

[13] Pöhlmann, A., *Arch. Dermatol. u. Syph.*, 1912–13, cxiv, 633.

[14] Gudzent, F., and Herschfinkel, *Strahlentherap.*, 1916, vii, 519.

[15] Hanzlik, P. J., and Tarr, J., *J. Pharmacol. and Exp. Therap.*, 1920, xiv, 221.

[16] von Oefele, F., *New York Med. J.*, 1913, xcvii, 78.

[17] Mines, G. R., *J. Physiol.*, 1910, xl, 327.

[18] Hébert, A., *Bull. Soc. chim.*, 1906, xxxv, series 3, 1299.

Germanium is absent from this list. We have been unable to find in the literature any references to therapeutic or physiological studies of this element or its compounds.

EXPERIMENTAL.

The germanium dioxide used in these experiments was supplied by Müller.[19]

It was found that the mature albino rat can withstand the subcutaneous injection of 180 mg. of a 0.4 per cent solution of germanium dioxide per kilo of body weight without fatal result or the appearance of any symptoms whatsoever of a toxic effect. The detailed report of these experiments is now in press.[20] That study was planned as a preliminary to the determination of the effect of germanium on the erythropoietic system because of the interest that has been attached to arsenic in this connection and because germanium occupies a place in the periodic system next to arsenic and in many of its reactions resembles this latter element.

For this study there were used two litters of mature albino rats. In one litter, 150 days old, there were three males and four females. In the other, which was about 175 days old, there were four males and four females. The females had been segregated before puberty and had never been pregnant. All the rats had from birth been on the same varied diet which was continued throughout the investigation. We thus had for the purposes of our experiment two groups of animals of two lots each in which constitutional variability was reduced to a minimum. The relatively low variability within the litter has been shown by Jackson[21] and King.[22] Each lot of rats was kept in a separate cage.

Two preliminary determinations, 7 days apart, were made of the erythrocyte and leucocyte counts of each rat of each lot in order to establish the normal values. One rat of each lot was reserved as control. Weekly counts of the cellular blood components were made of all the rats—controls and tests. The blood on which the counts were made was obtained by cutting off a bit of the tail for each determination. The blood was allowed to flow freely and the first 1 or 2 drops were discarded. This was done under light ether anesthesia as were the injections of the germanium dioxide solution.

---

[19] Müller, J. H., *J. Am. Chem. Soc.*, 1921, xliii, 1085.

[20] Hammett, F. S., Müller, J. H., and Nowrey, J. E., Jr., *J. Pharmacol. and Exp. Therap.*, 1922 (in press).

[21] Jackson, C. M., *Am. J. Anat.*, 1913–14, xv, 1.

[22] King, H. D., *Anat. Rec.*, 1915, ix, 751.

The test rats of the first group, both males and females, were injected with 1.2, 1.5, 1.8, and 2.1 mg. of germanium dioxide per kilo of body weight in sterile 0.4 per cent solution on the 8th, 11th, 15th, and 19th days of observation respectively. The solution was made at first slightly alkaline in order to facilitate the dissolving of the oxide. It was then brought back to neutrality by dilute hydrochloric acid. The rats of the second group were injected with ten times the amount given to the first group, on the 8th, 11th, and 15th days of observation. No injections were made on the 19th day because an increased coagulability of the blood had appeared which made difficult the taking of a proper sample. A total of 6.6 mg. per kilo of body weight was injected into the rats of the first group and of 45 mg. into the test rats of the second group.

Two more counts were made of the corpuscles, one on the 22nd and one on the 28th day of the experiment, the last count being made 11 days after the last injection in the first group and after an interval of 14 days in the second group. At this time the experiment was discontinued because the results were adequate, as shown by Tables I to V. The animals were anesthetized with ether and killed by crushing the spinal cord in the cervical region. All values represent the average of two counts made in two counting chambers. In Table V will be found the statistical data concerned with this study. The accepted criterion for observed differences to be statistically valid is that the probable error of the means must be contained at least twice and should be contained three times, for incontestable figures, in the difference between the means. The differences in the erythrocytes between the fore and after periods of the test rats and the differences between the control rats and the test rats are seen to be statistically valid.

The observations conclusively demonstrate that germanium dioxide causes a very marked increase in the number of red cells per cubic millimeter of blood. An increase in the erthyrocyte count of from 1

TABLE I.

The Influence of Small Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Male Albino Rat.

Day.	Control rat.		Test rats.				GeO$_2$ injected per kilo of body weight.
	R. B. C.	W. B. C.	No. 1.		No. 2.		
			R. B. C.	W. B. C.	R. B. C.	W. B. C.	
							mg.
1	7.45	7.2	8.17	9.6	7.61	8.3	
7	7.76	6.6	8.39	7.7	7.96	9.3	1.2*
10	—	—	—	—	—	—	1.5
14	7.57	6.5	9.26	8.6	8.42	7.9	1.8
18	—	—	—	—	—	—	2.1
21	7.78	7.1	9.60	7.8	10.37	8.6	
28	7.68	7.8	9.21	9.5	10.34	9.3	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

In Tables I to IV all values for the red cells are in terms of millions, all values for the white cells in terms of thousands.

TABLE II.

The Influence of Small Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Female Albino Rat.

Day.	Control rat.		Test rats.						GeO$_2$ injected per kilo of body weight.
	R. B. C.	W. B. C.	No. 1.		No. 2.		No. 3.		
			R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	
									mg
1	7.73	7.3	7.81	7.3	7.65	8.2	7.67	6.7	
7	7.98	7.7	7.79	7.2	7.86	8.4	7.74	7.4	1.2*
10	—	—	—	—	—	—	—	—	1.5
14	7.90	—	9.38	6.6	9.25	8.6	9.10	8.3	1.8
18	—	—	—	—	—	—	—	—	2.1
21	Killed by accident.		9.68	8.7	10.09	11.3	9.60	7.5	
28			9.34	7.5	9.58	10.3	8.92	8.6	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

TABLE III.

The Influence of Large Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Male Albino Rat.

Day.	Control rat.		Test rats.						GeO_2 injected per kilo of body weight.
			No. 1.		No. 2.		No. 3.		
	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	
									mg.
1	8.64	9.6	9.31	10.2	8.76	10.1	9.38	10.2	
7	8.61	10.4	9.30	12.2	8.70	10.1	9.38	10.1	12.0*
10	—	—	—	—	—	—	—	—	15.0
14	8.72	10.6	11.04	16.8	10.20	12.5	9.82	11.0	18.0
18	—	—	—	—	—	—	—	—	
21	8.95	9.2	10.96	12.2	10.16	8.6	10.79	9.1	
28	8.92	10.4	10.47	10.2	10.40	9.4	11.58	9.5	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

TABLE IV.

The Influence of Large Doses of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Female Albino Rat.

Day.	Control rat.		Test rats.						GeO_2 injected per kilo of body weight.
			No. 1.		No. 2.		No. 3.		
	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	
									mg.
1	7.70	13.9	7.38	7.1	9.52	7.0	8.69	8.7	
7	7.81	17.0	7.64	11.8	9.35	10.6	8.60	8.4	12.0*
10	—	—	—	—	—	—	—	—	15.0
14	7.97	20.0	9.20	9.4	10.54	9.1	9.36	9.6	18.0
18	—	—	—	—	—	—	—	—	
21	8.20	18.9	9.40	12.8	11.20	12.4	10.57	9.5	
28	8.01	13.1	12.31	10.3	10.66	10.9	11.56	8.9	

* The germanium dioxide solution was injected after the taking of the blood sample for the second count.

to nearly 5 million cells occurred in every one of the eleven rats which had had injections of this compound. No such response was exhibited by the control rats. Moreover, germanium dioxide does not produce a leucemia as an accompaniment of the erythrocytosis.

The combination of the fact that germanium dioxide is non-toxic and non-corrosive, with the fact that it produces such a marked increase in the number of the erythrocytes in the circulation of the healthy rat gives us the hope that this compound has a specific stimulating effect upon the erythropoietic tissue and will be found of clinical value.

An inspection of the tables will show that there is a tendency for the red count to increase most in those animals in which the original cell count is lowest. It is also evident that the small doses cause just as marked and significant a rise as do the larger doses.

TABLE V.

Statistical Data Concerned with the Study of the Influence of Germanium Dioxide on the Erythrocyte and Leucocyte Content of the Blood of the Albino Rat.

	Control rats.				Test rats.			
	Initial counts.		Subsequent counts.		Initial counts.		Counts after GeO_2 injections.	
	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.	R. B. C.	W. B. C.
Mean.	7,960,000	9,959	8,170,000	11,533	8,395,000	8,931	10,074,000	9,734
Standard deviation.	406,000	3,452	485,000	4,702	713,000	1,547	2,743,000	1,907
Probable error of mean.	97,000	823	104,000	1,057	103,000	222	322,000	223

Attention should be called to the fact that there occurred a slight but statistically valid increase in the erythrocytes of the controls in each series. This increase is attributed to the possibility of food contamination through excretion of germanium, and is supported by the fact that the effect was not observed in rats used as controls in a similar series of studies with arsenic.

At autopsy marked differences were found in the color of the liver and the bone marrow of the germanium-treated rats as compared with the controls. The liver of the control was a reddish tobacco-brown color, that of the test animals was a reddish purple. The bone marrow of the control albinos was the color of coffee with cream with here and there a slight tinge of red. That of the germanium-injected rats was maroon. No differences in the color of the spleens were detectable. A paper dealing with the histological findings will be published later.[23]

SUMMARY AND CONCLUSIONS.

Injections were made of a sterile 0.4 per cent solution of germanium dioxide into four lots of mature male and female albino rats. Exact conditions of control were maintained. To two lots there was administered in four doses at intervals of 4 days a total of 6.6 mg. of the oxide per kilo of body weight. To two lots there was given in three doses at like intervals a total of 45 mg. of the compound per kilo of body weight. In each lot there was one rat which served as a control and which did not receive any germanium. A preliminary period of observation was maintained as a further control during which two determinations of the erythrocyte and leucocyte counts of the blood were made 7 days apart on all the rats. Weekly counts were made on all the rats during the progress of the investigation which lasted for 4 weeks, during 3 of which the treated animals were under the influence of germanium. In the first group 11 days elapsed between the last injection and the last count. In the second group the interval was 14 days.

It was found that without exception all of the test rats responded to the germanium dioxide by a marked and sustained rise in the number of erythrocytes in the blood which ranged from 1 to nearly 5 millions.

These results are statistically valid.

There was an apparent tendency for the degree of effect to be related to the initial erythrocyte number, in that with a lower initial count there seemed to take place a greater rise, and *vice versa*.

There was no indication that the larger doses of germanium dioxide exerted a greater stimulating effect on the production of the resultant erythrocythemia than the smaller doses.

[23] Hammett, F. S., and Nowrey, J. E., Jr., *J. Exp. Med.*, 1922, xxxv (in press).

There is evidence that the effect is quick in making its appearance. The rise in the red cell count was found to occur within a week, and after but two injections of the oxide.

Indications were obtained that germanium dioxide tends to increase the coagulability of the blood.

Autopsy findings showed color changes in the liver and bone marrow.

We believe that germanium dioxide is an erythrocytogenic agent and we hope that fruitful results will come from its therapeutic application.

THE USE OF PHENOL RED AND BROM-CRESOL PURPLE AS INDICATORS IN THE BACTERIOLOGICAL EXAMINATION OF STOOLS.

BY ALAN M. CHESNEY, M.D.

(From the Department of Internal Medicine of Washington University School of Medicine, St. Louis.)

(Received for publication, July 18, 1921.)

There have been so many new media proposed during the past few years for the isolation from stools of organisms belonging to the typhoid-dysentery group that one hesitates to bring forward a new one. Believing that the indicators phenol red and brom-cresol purple possess distinct advantages over those formerly used in stool bacteriology, we have felt justified in pointing out their applicability in this particular domain of bacteriological technique.

A critical analysis of all the media advocated for the culture of stools is considered beyond the scope of this paper, but a brief mention of some of those more widely used does not seem amiss. In this country Endo's medium, prepared according to the author's formula or according to the modifications advocated by Kendall (1) or Robinson and Rettger (2), has been used perhaps more extensively than any other when a non-restraining medium was desired, although this medium has disadvantages which are well known to all workers in this field. The methylene blue-eosin agar of Holt-Harris and Teague (3) has found favor with some workers because it avoided some of the faults of the Endo medium. Meyer and Stickel (4) consider that either the medium of Holt-Harris and Teague or their own eosin-China blue medium prepared with peptic or tryptic digests is superior to the Endo agar, litmus agar, or Congo red agar "for the direct isolation and detection of dysentery bacilli from stool specimens." The extensive work of Krumwiede (5) and his coworkers upon the usefulness of brilliant green agar as a selective restraining medium has shown that this medium can be successfully applied in routine work when dealing with members of the typhoid-paratyphoid group. Teague and Clurman (6) advocate the use of a meat extract agar containing brilliant green and eosin as a selective medium for the isolation of members of the typhoid-paratyphoid group, and Meyer and Stickel[1] state that eosin and brilliant green used in connection with their peptic digest agar "permits the detection of a higher percentage of viable typhoid bacilli than any other one thus far introduced into bacteriologic technic." Kligler (7) finds that the Endo medium after the addition of sulfite reaches a hydrogen ion concentration ranging from pH 8.6 to 8.8 and states that this degree of alkalinity is unfavorable to the growth of the Shiga bacillus. He also advocates the use of neutral red with the brilliant green plate of Krumwiede and considers this the best among several indicators that he tried. Kligler thinks also that the reaction of the medium has a marked influence upon the restraining activity of the brilliant green. It may be mentioned at this point that Krumwiede (8) objects to the use of eosin in connection with brilliant green because of the resultant eye strain from the irritating color produced by this combination. This objection would seem to hold good only where large numbers of plates are to be examined by one worker. Morishima (9) has recently advocated the use of a combination of phenol red and China blue as an indicator for lactose plates in the culture of stools.

---

[1] Meyer and Stickel (4), p. 67.)

In this communication it is proposed to record our experience with the use of phenolsulfonephthalein (phenol red) and dibromo-ortho-cresol-sulfonephthalein (brom-cresol purple) as indicators in stool bacteriology. To Clark and Lubs (10), largely, belongs the credit for the introduction of these indicators into bacteriological technique. They suggest that these indicators might be serviceable in the preparation of indicator plates (11), but so far as our survey of the literature has gone we have not been able to find any reference to the use of these indicators in stool bacteriology except in the case of the combination of phenol red with China blue, as advocated by Morishima.

Method.

In studying these indicators to determine their availability for stool work the basis of the medium used was a 1.5 and a 3 per cent beef extract agar. To this agar were added varying amounts of lactose from sterile solution and varying amounts of indicator, in order to determine the optimum amount of each of these ingredients. The reaction of the medium also was varied in order to ascertain the pH at which sharpest color differentiation was had. Plates were seeded with mixtures of *Bacillus coli* and members of the typhoid-dysentery group, also with stools inoculated with members of the latter. When the optimum amounts of the constituents had been ascertained, brilliant green was added to the agar in the proportions recommended by Krumwiede, and the degree of restraining activity noted as compared with controls consisting of agar with brilliant green alone and agar with the indicator alone.

RESULTS.

In the case of both indicators a 3 per cent beef extract agar cleared with egg to which 1 per cent lactose had been added from sterile 20 per cent solution gave the best results. With the thicker agar there was less diffusion of acid. Thick plates, about 20 cc. of agar to a plate, were poured. We shall discuss the two indicators separately.

Phenol Red.—The optimum reaction of the agar, if this indicator is to be used, is a hydrogen ion concentration represented by a pH of 7.6 to 7.8. The optimum amount of indicator is 10 cc. of a 0.04 per cent aqueous solution for every 100 cc. of agar. The indicator may be sterilized by autoclaving. Plates poured from agar with a pH of 7.6 to 7.8 and containing the amount of indicator mentioned will assume on cooling a color best described as ranging between salmon pink and old rose. Such plates are clear, and upon them lactose-fermenting organisms produce vivid greenish yellow colonies with a surrounding zone of green. The typhoid bacillus, the paratyphoid bacillus (A and B), and the dysentery bacilli (Shiga, Flexner, and Hiss Y), all produce pink colonies. These organisms evidently produce alkali, for the surrounding medium assumes a decidedly deeper pink than the uninoculated portions of the plate. This alkali production with consequent intensification of the pink color of the medium in the neighborhood of the typhoid-dysentery colonies is a helpful factor in the differentiation of the latter. In thickly seeded plates the diffusion of the acid in the vicinity of the lactose-fermenting colonies is often sufficient to mask this change in the direction of alkalinity exhibited by the typhoid-dysentery colonies, but where good distribution is obtained the color change is most striking. Even in thickly seeded plates where typhoid and colon colonies abut upon one another, a difference in the color of the two types of colonies is observable. The colon colonies assume a bright green or yellow-green color and are opaque, whereas the typhoid colonies in such cases are more translucent and possess a bluish green color. The dye does not mask the corrugated, woolly appearance of the typhoid colonies which is characteristic of them and upon which Krumwiede lays great stress in their recognition. On the contrary, we are inclined to feel that the dye perhaps enhances this characteristic. We have had no opportunity to study freshly isolated strains belonging to the dysentery group, and it is possible that such strains may be inhibited by this indicator; however, it does not inhibit the growth of freshly isolated strains of *Bacillus typhosus* or *paratyphosus*. The indicator itself is not reduced by the bacteria. We have found it very satisfactory when used in connection with sugar broth or Russell's double sugar.

Brom-Cresol Purple.—In our experience this indicator, when used in connection with lactose plates, gives a sharper color differentiation than does phenol red and hence is preferable to the latter. It is purple in alkaline solution, changing to yellow in the presence of acid. The color change takes place in the zone of pH 5.2 to 6.8. The optimum reaction of the agar if this indicator is to be employed is a pH of 7.2 to 7.4. The optimum amount of indicator to use is 5 to 8 cc. of a 0.04 per cent aqueous solution for every 100 cc. of agar. This solution may be sterilized in the autoclave. Plates poured from agar with a reaction of pH 7.2 to 7.4 and with the amount of indicator mentioned assume a deep blue color and are clear. Upon these plates the lactose-fermenting organisms produce greenish yellow colonies with a yellow zone, and the non-lactose fermenters produce bluish colonies. The typical appearance of the typhoid colonies is not obscured by this indicator. Even on thickly seeded plates the colonies of the non-lactose-fermenting organisms tend to preserve a bluish appearance, being bluish green rather than a brilliant yellow, the color that the colon bacillus colonies assume. It is not an infrequent experience on thickly seeded plates to see a round yellow colon colony with a wedge-shaped bluish green sector occupying a portion of the circle and representing a typhoid colony which has refused to be masked by the larger, acid-producing colony of colon bacilli. This indicator did not show any restraining influence upon any of our laboratory cultures of *Bacillus dysenteriæ*, including the Shiga variety. Whether or not it would exercise any restraint on freshly isolated cultures we are not able to say.

Combination of Phenol Red or Brom-Cresol Purple with Brilliant Green.—To judge from our rather limited experience either indicator may be used along with brilliant green in the amounts advocated by Krumwiede. We have had an opportunity to study only one sample of brilliant green, the source of which was unknown but which was active in its restraining power upon *Bacillus coli*. Neither indicator, so far as could be determined, exercised any inhibiting effect upon this restraining power of the brilliant green. The only change noticed was in the color of the plates, the phenol red-brilliant green plates being brownish red in color and the brom-cresol purple-brilliant green plates being bluish purple. The characteristic color changes produced by the lactose-fermenting colonies were not altered by the brilliant green.

DISCUSSION.

Since the introduction of lactose indicator plates for the bacteriological examination of stools for the detection of members of the typhoid-dysentery group, numerous attempts have been made to find a medium which was entirely satisfactory. As pointed out by Hiss and Zinsser (12) the best results are apt to be attained when an individual becomes thoroughly familiar with one type of medium and restricts himself to it. Endo's medium has the disadvantage of tending to vary with different batches, some being satisfactory and others useless. There can be little question but that the selective restraining media have been highly successful in skilled hands when large numbers of stools have had to be examined. Few workers, however, in carrying out routine examinations with such media, are willing entirely to dispense with a non-restraining medium such as the Endo plate, but prefer to use it in addition as a control. This is particularly apt to be the case in laboratories serving hospitals, where the proportion of stool cultures to other bacteriological examinations is not great and where the workers cannot be expected to attain the degree of skill in stool bacteriology which is attained by those carrying out an extensive series of bacteriological examinations of stools. For the less experienced worker, let us say, a non-restraining medium is essential, if only as a check, and a medium which does not obscure the characteristic appearance of the typhoid colonies and at the same time shows a sharp color differentiation should be satisfactory for the purpose.

It is because we believe that the indicators phenol red and brom-cresol purple may be successfully employed with lactose to give a pected by Hall and Whipple but not conclusively proven. Our experiments in normal dogs under controlled conditions appear convincing when exposures given over the thorax are contrasted with the same exposures over the abdomen. For example, a unit dose (350 milliampere minutes) given over the thorax (abdomen shielded) causes no clinical disturbance in the dog. A subsequent exposure to the same unit dose in the same dog given over the abdomen (thorax shielded) will cause fatal intoxication and death in 4 days. For a normal 30 to 40 pound dog, we may say that 350 milliampere minutes given over the abdomen is a minimum lethal dose.

The outstanding abnormalities in the abdominal exposures are the remarkable necroses observed in the small intestine. Large areas may show complete necrosis and disappearance of the intestinal epithelium covering the villi and lining the crypts. The villi are left as naked polyps made up of stroma, vessels, and a few wandering cells. The inflammatory reaction is not as intense as would be expected under such conditions with complete removal of the covering epithelium. This important point will be discussed in detail in a subsequent paper.

Regaud, Nogier, and Lacassagne (5) described the chronic gastrointestinal lesions in dogs and noted especially gastric atrophy and intestinal perforation. Fromme (2) noted especially the clinical symptoms of gastrointestinal disturbance in guinea pigs and mice. Denis, Martin, and Aldrich (1) noted in rabbits the toxic effect of abdominal radiation and certain abnormalities in the intestines which they state are secondary to an unknown toxic factor.

Method.

The procedure is identical in all experiments, unless otherwise indicated. Normal dogs are used throughout and are kept in standard metabolism cages which have sharply pitched floors to facilitate the collection of urine. To insure the acidity of the cage collections, about 1 cc. of glacial acetic acid is added to the cage collecting bottle at the beginning of each 24 hour collection. The dogs are catheterized daily at 10 o'clock. The cage collections, cage washings, bladder washings, and catheterized urine are mixed and diluted to a unit volume—usually 2 liters. The total nitrogen is then done in duplicate by the Kjeldahl method. About 300 cc. of warm water is given daily by stomach tube as a routine procedure after the dog is catheterized. Water is available at all times in the cage.

Dogs are given a subcutaneous injection of morphine shortly before exposure to radiation. A medium hard Coolidge tube is used with the target (anode or anticathode) set at a constant distance of 10 inches from the skin. At this distance the effective radiation for this machine covers a circle approximately 6½ inches in diameter. The current strength used is either 7.5 or 8.0 milliamperes. The E.M.F. varies considerably in different experiments from 85 kilovolts to 103 kilovolts. The spark-gap backing up these voltages varies also, measuring from 8½ to 10 inches between sharp points. The amount of radiation is calculated as the product of the current strength in milliamperes and the time of exposure in minutes and is expressed as milliampere minutes. In all these experiments the standard autotransformer x-ray equipment of the University of California Hospital is used. Aluminum filters (2 mm.) are used to cut out the softer rays. The various parts of the dog were effectively protected from radiation, when it was so desired, by sheets of lead-foil approximately 2 mm. thick, or by lead "rubber," approximately 5 mm. in thickness.

The body of the dog is marked off roughly into two parts by a transverse line at the level of the xiphisternum. The part between this line and the base of the neck is designated as the thorax, that below as the abdomen. Each of these parts is then divided into an upper and lower half. Each upper and lower segment of the abdomen or thorax is radiated from the lateral and midventral aspects. Thus the ventral portion of the abdomen or thorax is exposed to radiation over six different areas whose contiguous edges overlap somewhat. Usually one exposure is given over the middorsal region of the thorax or abdomen, causing the total dosage to be divided up into seven parts, which, however, are spread rather diffusely over the part of the body involved. All dogs are sacrificed under chloroform or ether anesthesia by bleeding.

EXPERIMENTAL OBSERVATIONS.

Some of our typical experiments are tabulated below and make several points quite clear. However, we wish to refer to other experiments of similar nature given in subsequent papers of this series. These experiments are all in agreement and indicate that enormous doses of Roentgen rays may be given over the thorax without causing any clinical reaction in a normal dog. Dogs have been given amounts of radiation varying from 300 to 512 milliampere minutes over the thorax without any clinical symptoms. One dog (No. 19–117) was given 350 milliampere minutes over the thorax on three separate occasions with intervening periods of 2 to 3 weeks; all this without any apparent cumulative effects or clinical reaction. There is usually a slight transient leucopenia and there may be a very slight rise in total nitrogen excretion. After 2 or 3 weeks, the skin shows loss of hair and pigmentation as a result of the exposures. The ribs of the animal examined at autopsy show a considerable loss of marrow cell elements. We have been able to recognize no other results from these large doses of x-rays over the thorax.

Dog 19–78 (Table I) is a typical example of a complete experiment to show that a unit dose of x-rays is clinically inert over the thorax but lethal when given over the abdomen. The thorax dose causes a definite leucopenia but no clinical disturbance. That the second dose does not cause as much of a leucopenia, we believe, may be due to the tissue injury in the small intestine.

The radiation over the abdomen causes a typical clinical reaction and the review of this case will suffice for all subsequent experiments. This clinical reaction is remarkably constant and is described in detail below (Dog 19–78). The latent period of 24 to 36 hours is noted as usual and will be discussed later. The rise in total urinary nitrogen is not great but is significant. The figure for the last day includes a certain amount of fecal contamination.

Dog 19–78. Clinical History and Autopsy (See Table I).

Jan. 27. The dog is isolated on fasting diet. The animal is in good condition and quite active.

Jan. 29. The abdomen is covered with lead-foil well up to the level of the diaphragm. The thorax is exposed in seven areas, two midventral, two on each side, and one over the midscapular region. Each area exposed covered a patch of skin approximately 5 inches square. Morphia ($\frac{5}{8}$ gr.) is given to keep the dog quiet during the exposure. The current strength is 7.5 milliamperes at an E. M. F. of 100 kilovolts with the spark-gap set at 9 $\frac{9}{16}$ inches. 2 mm. of aluminum filter is used and the tube is set at a target-skin distance of 10 inches.

TABLE I.

Roentgen Radiation.

Unit Dose Given over Thorax and Subsequently over Abdomen.

Dog 19–78. Adult, female.

Date.	Weight.	White blood corpuscles per c. mm.	Urine.		Diet.	Remarks.
			Volume.	Total N.		
	lbs.		*cc.*	*gm.*		
Jan. 28	26.0	—	—	—	Water.	Good condition.
" 29	26.0	7,600	—	—	"	
Jan. 29	X-rays, 350 milliampere minutes, given over thorax. Abdomen shielded.					
Jan. 30	25.8	8,000	—	—	Water.	Dog normal.
" 31	24.5	4,100	—	—	"	
Feb. 1	23.8	4,600	—	—	"	Dog normal.
" 2	23.3	4,600	—	—	"	
" 3	23.0	—	—	—	Mixed.	Dog normal.
" 8	27.0	5,400	—	—	"	
" 21	28.3	7,400	—	—	"	Dog normal.
	Experiment ended.					
Mar. 15	28.9	7,800	—	—	Water.	Dog normal.
" 17	27.8	7,600	260	2.74	"	
" 18	27.3	8,000	305	2.68	"	Dog normal.
Mar. 18	X-rays, 350 milliampere minutes, given over abdomen. Thorax shielded.					
Mar. 19	26.8	6,400	290	2.83	Water.	Dog normal.
" 20	26.6	7,000	265	2.74	"	Inactive.
" 21	25.9	7,200	240	3.00	"	Vomitus and diarrhea.
" 22	25.0	3,900	—	5.63	"	Moribund.
	Chloroform anesthesia and autopsy at once.					

Jan. 30. Dog remains normal during entire experiment (see Table I). There was a slight transient leucopenia. Dog was on mixed diet until the second experiment, also given in Table I.

Mar. 15. The dog is active and in good condition.

Mar. 18. Dog given ½ gr. of morphia 1 hour before exposure to radiation. The thorax and head and neck are thoroughly screened with lead-foil down to the tip of the xiphisternum. Seven areas are exposed, two midventral, two on each lateral surface, and one midlumbar. The current strength is 7.5 milliamperes at an E. M. F. of 95 kilovolts, backed by a 9½ inch spark-gap. The radiation is filtered by 2 mm. of aluminum. The skin-target distance is 10 inches. There are no immediate after effects from exposure to 350 milliampere minutes.

Mar. 19. The dog is bright and active.

Mar. 20. Slight evidences of intoxication are manifested by the dog—one soft, yellow, watery stool.

Mar. 21. The intoxication becomes marked in the afternoon. The diarrhea has progressed from brownish, watery material to tarry, black mucous or bloody mucous material with a very foul odor. Vomitus increases in amount and frequency during the day.

Mar. 22. The animal is very weak and dull. In the afternoon, the dog is completely prostrated. The stools have become very frequent and of large volume. The pulse is irregular and thready. The temperature has risen to 40.2°C. The respirations are rapid and irregular. The animal is practically moribund and is sacrificed under chloroform anesthesia.

Autopsy.—Performed immediately after death by chloroform. The hair over the thorax is thinned. It is absent over the sternum where the skin is somewhat pigmented from previous exposures to radiation. Pleural cavity, heart, and lungs are normal. Peritoneal surfaces are normal. Spleen is somewhat granular in appearance, hard and firm, normal in size. On section, the trabeculæ stand out. Mesenteric glands slightly enlarged and moist—somewhat pinkish in color. Kidneys normal in size and appearance. Bladder contracted; it shows a few ecchymoses in its mucosa (catheter). Liver is normal. Adrenals are somewhat enlarged; apparently normal except for a small necrotic mass in the left adrenal.

Stomach contains gas and about 20 cc. of a bile-stained fluid. The mucosa is pale and normal.

Duodenum is somewhat thick-walled and spastic. The lumen contains many large, round worms and a small amount of reddish-black mucus. Beginning sharply at a point approximately an inch distal to the *pyloric valve*, the mucosa of the *duodenum* presents a dark red glassy appearance. On this background run several longitudinal folds with bright red hemorrhagic crests. Numbers of small bright red petechiæ show up between these plicæ on the dark, glassy surface,

The reaction in the *jejunum* seems less intense, for here the mucosa is paler and the plicæ are not so conspicuous though they can be distinctly made out.

Ileum contains a small amount of reddish-black mucous material similar to the stools, and a few round worms. The intestinal wall is thin and rather flaccid and on close inspection the intensely hemorrhagic mucosa surface seems to have been denuded of most of the villi as the velvety appearance is lacking. The longitudinal folds with bright red hemorrhagic crests are conspicuous. Peyer's patches seem to be stripped of their villous covering so that they show up readily as speckled oval patches. The ileocecal valve marks an abrupt end of this extensive injury.

Colon mucosa is pale, though a few longitudinal, hemorrhagic folds are present. The walls are spastic and the lumen contains a small amount of reddish fluid.

Histological Sections.

Lungs, heart, liver, pancreas, kidneys, bladder, uterus, and stomach are negative. The mesenteric lymph nodes are slightly hemorrhagic and contain some pigment. The spleen is moderately atrophic and contains pigment. In the ovary the ova are apparently dead, though nucleoli and chromatin remnants are still present within the nuclear membrane.

Pyloric duodenum: a section shows the transition from normal intact pyloric villi to collapsed and denuded villi with remnants of dead epithelium and some regenerating epithelial cells undergoing mitosis. Some areas have escaped injury.

Jejunum: destruction and disappearance of the epithelium of villi and crypts; a few crypts remain, otherwise the normal structure is gone. The epithelium around these crypts shows mitotic figures and these are apparently regeneration forms. Large pale epithelial cells stick out into the lumen of the crypts. Their cytoplasm is finely meshed and is decidedly eosinophilic. The nuclei are rather large and pale and frequently contain mitotic figures. A large amount of hemorrhage has occurred in the submucosa, and blood vessels are occasionally found plugged with thrombi of fibrin and platelets. On some areas a very thin sheet of epithelial cells can be seen making an attempt to recover the collapsed villi.

The *ileum* shows extreme injury, with the disappearance of the greater part of the crypt and villous epithelium. Strenuous attempts at regeneration are being made by remnants of the epithelium which shows many mitotic figures and is found clumped in little groups or sheets as if these groups were descendants of one or two surviving cells of the original epithelium.

The *colon* shows some mucoid degeneration and slight necrosis of the tips of a few villi, with evidence of repair.

Dog 19–85. Clinical History and Autopsy (See Tables II and IV).

Dog 19–85 received 300 milliampere minutes over the abdomen (Table IV) which was followed by diarrhea and vomitus and prostration. She recovered. 1 month later she was given 350 milliampere minutes over the thorax with no effect (Table II). A month later (2 months after the first exposure) she was given 350 milliampere minutes over the abdomen with fatal result (Table II).

Mar. 2. Dog fasting since Feb. 28.

Mar. 4. Dog given ½ gr. of morphia 1 hour before exposure to radiation. Lower part of body screened up to xiphisternum by lead-foil 2 mm. thick; 350 milliampere minutes of radiation divided up into the usual seven areas. A current strength of 7.5 milliamperes is used at 103 kilovolts and backed up by a 9⅞ inch spark-gap. A 2 mm. aluminum filter is used. Skin-target distance 10 inches. The total time of exposure is 46.6 minutes. There are no apparent after effects from the x-ray exposure.

Mar. 5 to 8. The dog remains clinically normal.

TABLE II.

Roentgen Radiation.

Unit Dose over Thorax Negative but Subsequent Unit Dose over Abdomen Lethal.

Dog 19–85. Adult, female.

Date.	Weight.	White blood corpuscles per c. mm.	Urine.		Diet.	Remarks.
			Volume.	Total N.		
	lbs.		*cc.*	*gm.*		
Mar. 2	24.7	—	—	—	Water.	Good condition.
" 3	24.4	7,800	355	2.04	"	
" 4	24.1	6,800	310	1.71		Dog normal.
Mar. 4	X-rays, 350 milliampere minutes, given over thorax. Abdomen shielded.					
Mar. 5	23.8	6,600	290	1.90	Water.	Dog normal.
" 6	23.4	8,000	325	1.79	"	" "
" 7	23.2	5,400	325	1.96	"	" "
" 8	22.9	6,800	—	—	"	
" 9	22.8	—	—	—	Mixed.	
	Experiment ended.					
Mar. 30	25.0	—	—	—	Water.	Dog normal.
" 31	24.6	—	440	2.07	"	
Apr. 1	24.1	7,400	325	1.90	"	Dog normal.
Apr. 1	X-rays, 350 milliampere minutes, given over abdomen. Thorax shielded.					
Apr. 2	23.4	7,300	300	2.13	Water.	Dog normal.
" 3	23.1	5,800	290	1.90	"	" "
" 4	22.8	7,200	240	2.07	"	Diarrhea and vomitus.
" 5	22.1	5,400	275	3.16	"	Severe intoxication.
	Ether anesthesia and autopsy at once.					

Mar. 30. Dog has remained normal since the exposure over the thorax. There is some depilitation and pigmentation of previously exposed areas. Dog fasting for past 3 days.

Apr. 1. Animal given ¼ gr. of morphine 1 hour before exposure; 350 milliampere minutes of radiation spread diffusely over the abdomen in six areas, all ventral. Each area averages approximately 25 square inches. The current strength is 7.5 milliamperes at an E. M. F. of 90 kilovolts, backed up by a 9 inch spark-gap. A 2 mm. aluminum filter is used. Skin-target distance 10 inches. There are no clinical after effects which could be attributed to the radiation.

Apr. 2 and 3. The animal is active and lively.

Apr. 4. There is quite a large volume of yellow, semifluid feces and some bile-stained vomitus throughout the morning. Intoxication becomes more marked in the afternoon. The pulse is regular, but of low tension.

Apr. 5. Greenish-brown, semifluid stools and bile-stained vomitus appear regularly throughout the day. In the afternoon, the stools are tarry and vile smelling. In general, the stools appear to contain digested or partly digested red blood cells, mixed with mucus. The vomitus at no time contains any blood, but is made up of a rather opalescent fluid containing thick, stringy mucus, which at times is bile-stained. The animal is markedly intoxicated, especially during the afternoon. Dog is able to walk but quite weak and ceases to respond voluntarily. The animal is not moribund and might survive, but in all probability would die during the night. Ether anesthesia and autopsy at once.

Autopsy.—Body fat well preserved. Pleural cavity and thoracic viscera are negative. Peritoneal cavity contains no fluid and the peritoneal surfaces are normal. Liver, spleen, pancreas, adrenals, kidneys, and genitourinary tract are negative.

Stomach contains about 20 cc. of bile-stained, frothy mucus, corresponding to the vomitus of the past 2 days. The mucosa is pale and apparently negative.

Small intestine is somewhat spastic and contains a small amount of greenish-brown mucous material.

Longitudinal folds with hemorrhagic crests are conspicuous in the duodenum. These fade out and disappear in the upper jejunum. In both the duodenum and ileum, there are patches or clusters and streaks of tiny ecchymotic specks in the mucosa. The superficial necrosis is not very extensive but stands out in contrast to the normal patches of mucosa in the jejunum and upper ileum, which are pale and velvety and apparently undisturbed.

Colon is negative.

Histological Sections.

Lungs, liver, pancreas, kidneys, adrenals, bladder, and uterus are negative. Ovary: the ova are dead, the nuclear material is broken up, and the cytoplasm is foamy. Spleen: atrophy of the Malpighian bodies and pulp elements; a few fragmented nuclei are seen. Stomach: normal. Duodenum: irregular and patchy injury of villus structures is apparent. In injured areas, there are evidences of mitosis and rapid growth of epithelial cells and some infiltration of the area by polymorphonuclear leucocytes. The irregularity of the injury is especially noted in the sections from the jejunum.

Ileum shows extensive injury and destruction of villous epithelium. The epithelium covering the villi and crypts has disappeared except for some rapidly growing epithelial cells apparently regenerating in an effort to cover the denuded areas. Polymorphonuclear and mononuclear leucocytes are rather numerous at the bases of the crypts.

Colon shows a certain amount of mucoid degeneration and here and there an occasional focus of polymorphonuclear infiltration especially under the areas where the epithelium is injured.

Dog 19–101. Clinical History and Autopsy (See Table III).

Feb. 17. Dog is active and in good condition.

Feb. 21. ¼ gr. of morphia is given 1 hour previous to the radiation; 117 milliampere minutes of radiation are given with a current strength of 7.5 milliamperes, at an E. M. F. of 103 kilovolts. It was then found necessary to give the remaining 233 milliampere minutes at a current strength of 10 milliamperes with an E. M. F. of 90 kilovolts. 2 mm. of aluminum filter used, spark-gap of 9 inches between sharp points. The skin-target distance is 10 inches.

Apr. 7. Dog is exposed to 450 milliampere minutes of radiation over the abdomen and sacrificed under ether anesthesia on Apr. 11.

TABLE III.

Roentgen Radiation.

350 Milliampere Minutes over Thorax Negative.

Dog 19–101.

Date.	Weight.	White blood corpuscles per c. mm.	Urine.		Diet.	Remarks.
			Volume.	Total N.		
	lbs.		*cc.*	*gm.*		
Feb. 17	34.0	8,000	—	—	Water.	Dog normal.
" 20	31.0	—	—	—		
" 21	30.4	7,800	400	3.03		
Feb. 21	X-rays, 350 milliampere minutes, over thorax. Abdomen shielded.					
Feb. 22	30.1	7,300	415	3.02	Water.	Dog normal.
" 23	30.0	7,900	605	2.86	"	
" 24	29.1	5,600	565	2.69	"	
" 25	28.8	7,300	605	2.52	"	
" 26	28.1	7,600	408	2.49	"	
" 27	28.1	7,400	370	2.24	"	Dog normal.
" 28	27.8	6,900	335	2.21	"	
	Experiment ended.					
Apr. 11	31.3	7,600	—	—		
	Ether anesthesia and autopsy at once.					

Autopsy.—The pleural and pericardial surfaces are moist and shiny. The heart and lungs are negative. The other changes do not concern the experiment given in Table III.

Dog 19–50. Clinical History and Autopsy.

To be contrasted with Table III; a control experiment over the abdomen.

Feb. 10. Dog active and normal; fasting.

Feb. 11. ¼ gr. of morphia 1 hour previous to radiation; 340 milliampere minutes are spread over the abdomen in the usual seven areas; screening of the thorax and lower pelvis with lead-foil. A current strength of 7.5 milliamperes at 97 kilovolts is backed up by a 9½ inch spark-gap; 2 mm. of aluminum filter the radiation. The skin-target distance is 10 inches.

Feb. 12. The dog is lively and active. A small amount of white frothy vomitus and some fluid brown feces appear on this day.

Feb. 13. There is evidence of slight intoxication. The pulse tension is low. The vomitus is of large volume, as much as a pint in an hour, and is deeply bile-stained. The diarrhea is frequent but scanty in amount and consists mostly of blood. At times, it is black, thick, and tarry. The dog is quite weak.

Feb. 14. The intoxication is quite severe. The animal is weak. The pulse is of low tension and quite irregular. The vomitus is large in amount and deeply bile-stained. The diarrhea is profuse and frequent. At times, the fecal material seems to be made up practically of pure blood and contains many clots. Again it may be tarry and vile smelling. The dog is comatose and is sacrificed under ether anesthesia. Autopsy at once.

Autopsy.—Thoracic viscera and serous surfaces are negative. The liver, spleen, adrenals, kidneys, lymph nodes, and genitourinary tract are negative. The pancreas shows a few small fat necroses.

Stomach: filled with a bloody mucous material; mucosa pale and normal.

Small intestine is spastic and contains a small amount of bloody fluid. The mucosa is a dull red, darker in the duodenum, lighter in the ileum. Longitudinal folds with hemorrhagic crests are evident in some sections, especially the paler sections of the lower jejunum, but they are masked in the duodenum by the intense dark dull red of the mucosa there. The intestinal wall is thin, apparently due to much loss of substance in the mucosa. This is especially marked in the ileum.

Colon and *cecum* contain a bloody fluid. Their mucosa is in general pale.

Histological Sections.

Liver, kidneys, adrenals, uterus are normal. Spleen shows some pulp atrophy and prominence of the reticulum. Pancreas contains a few fat necroses in one section, otherwise it is normal. In the ovary, the chromatin network of the ova is broken up, though the nucleoli are still present. The ova in general appear abnormal and seem to be degenerating.

Stomach and *colon* are normal.

Small intestine presents the usual picture of necrosis and destruction of villous and crypt epithelium, regeneration of epithelium with mitotic figures, and infiltration of polymorphonuclears into the bases of the crypts.

In the *ileum* the villi show only a naked reticulum with a few groups of regenerating epithelial cells and the lymph follicles. The blood vessels in the mucosa are frequently thrombosed.

The *mesenteric lymph nodes* are filled with red blood cells and polymorphonuclears and phagocytic cells containing cell debris and pigment.

TABLE IV.

Roentgen Radiation.

Sublethal Dose over Abdomen.

Dog 19–85.

Date.	Weight.	White blood corpuscles per c. mm.	Diet.	Remarks.
	lbs.			
Feb. 4	25.0	8,000	Water.	Dog normal.
Feb. 4	X-rays, 300 milliampere minutes, over abdomen. Thorax shielded.			
Feb. 5	24.5	6,000	Water.	Dog normal.
" 6	24.0	8,000	"	
" 7	23.5	3,400	"	Inactive.
" 8	23.3	8,400	"	Vomitus and diarrhea.
" 9	23.0	5,800	"	No diarrhea or vomitus.
" 10	23.0	7,800	"	Clinical improvement.
" 11	22.5	5,400	Mixed.	
" 20	25.0	7,400	"	Good condition.

Dog 19–85. Clinical History (See Table IV).

Feb. 3. Dog active and in good condition; fasting.

Feb. 4. ¼ gr. of morphia given 1 hour before radiation. The upper part of the body above the level of the xiphisternum is screened by 2 mm. of sheet lead-foil. Exposures made over the abdomen and pelvis in seven parts. The current strength is 7.5 milliamperes at an E. M. F. of 100 kilovolts, backed up by a 9½ inch spark-gap. The radiation is filtered by 2 mm. of aluminum. The skin-target distance is 10 inches. The total exposure time is 40 minutes—300 milliampere minutes. The dog is clinically unaffected by the exposure to radiation.

Feb. 5 to 7. Dog normal.

Feb. 8. The animal shows a slight degree of intoxication. The vomitus which appears is mostly a clear frothy mucus. Diarrhea is brownish and scanty.

Feb. 9. Mild intoxication; no diarrhea or vomitus.

Feb. 10. From this date, recovery to normal is rapid.

DISCUSSION.

At the very start of this discussion, we wish to point out the fact that these experiments concern normal dogs and not human beings suffering from disease, for example leucemia, cancer, and so forth. We feel that these experiments are less complicated for this very reason and may give us the opportunity to correct mistaken impressions gotten from the complex reactions which may result from Roentgen radiation of a diseased human being.

Our negative results of thorax radiation (up to 512 milliampere minutes) are of interest in ruling out convincingly certain of the body cells which have been suspected as causative factors in the general Roentgen ray intoxication. For example, these enormous doses given over the normal thorax must injure more bone marrow cells than do the abdomen exposures. The ribs and thoracic vertebræ surely contain more units of red marrow than do the lumbar vertebræ. Examination of the rib marrow some days after an exposure shows almost complete disappearance of red marrow cells, so we know that this reaction has taken place, yet there is not a trace of any clinical disturbance. This rules out the red marrow as a factor in the characteristic systemic disturbance noted in our experiments.

These same arguments apply to lymphatic tissue. The lymph glands in thorax and abdomen usually show some loss of cells but not to any very great extent. It is significant that Peyer's patches in the intestine may show only moderate injury when the epithelium covering the lymph follicles and lining the crypts has completely vanished due to the x-ray injury. In our experiments, therefore, the intestinal epithelium is much more sensitive to injury than are the cell elements of the lymph gland and Peyer's patch.

The spleen of the dog is not profoundly affected by the Roentgen radiation. For one thing, it is not as cellular as the human spleen, but we feel that the normal spleen cells are not easily destroyed by the x-rays. After large exposures we note a moderate atrophy of the spleen with shrinkage of the Malpighian corpuscles, but plenty of lymphocytes remain even after intensive radiation.

The thorax exposures, of course, cover the heart and lungs, which may be dismissed without comment. This area also includes a large part of the *circulating blood*. We conclude, therefore, that the x-rays have no injurious effect upon the circulating blood cells.

The thorax exposures therefore rule out several factors which at one time or another have been thought to be responsible for the general systemic intoxication—red bone marrow, lymphatic tissue, and circulating blood. We may now consider the various tissues in the abdomen which may be responsible for the fatal reaction following large doses of the x-rays.

The kidneys are very slightly disturbed, as is shown in a paper of this series (McQuarrie and Whipple (4)).

The liver is not concerned and we believe can be ruled out without question. There is no histological evidence of injury, but more than that, there is no evidence of functional disturbance. Dogs with bile fistulæ show no disturbance in function and no change in the level of bile acid and bile pigment output.[1]

The pancreas and adrenals show no histological evidence of injury by the x-rays. We know of no functional disturbance which may be attributed to these structures.

The gastrointestinal tract is remarkably sensitive to the Roentgen rays. In subsequent experiments, we shall review the remarkable ulcers which can be produced in the stomach, but as a whole, the stomach and colon are considerably more resistant than the small intestine.

The *epithelium of the small intestine* from the pylorus to the ileocecal valve is remarkably sensitive to the Roentgen rays. These doses used will cause necrosis of this epithelium with practically complete disappearance. We believe the symptoms of intoxication and the final lethal poisoning are due to this injury of the intestinal epithelium. We need not refer to the severe intoxication which develops following a functional disturbance of this same epithelium (intestinal obstruction). We may infer that the x-rays may produce sublethal injury to this intestinal epithelium which may be responsible for a variety of disturbances associated with gastrointestinal symptoms.

---

[1] Experiments to be published elsewhere.

SUMMARY.

Roentgen radiation of the thorax (abdomen shielded) in dogs, even with large doses (up to 512 milliampere minutes), gives no clinical evidence of intoxication. There may be a transient leucopenia and a slight rise in urinary nitrogen.

Roentgen radiation of the abdomen (thorax shielded) in dogs, with a dose of 350 milliampere minutes, will almost certainly cause a fatal intoxication. Smaller doses may be survived but usually with signs of gastrointestinal intoxication.

This lethal intoxication due to abdominal radiation presents a remarkably uniform clinical and anatomical picture. There is a latent period of 24 to 36 hours, during which the dog is perfectly normal clinically. The 2nd day usually shows the beginning of diarrhea and perhaps some vomitus. The 3rd and 4th days show progressive intoxication with increasing vomiting and bloody diarrhea until the dog becomes stuporous. Death is almost always on the 4th day.

Anatomically the only lesions of significance are to be found in the small intestine. The epithelium of the crypts and villi shows more or less complete necrosis, and this condition may involve almost all of the small intestine. The epithelium may vanish completely except for a few cells here and there which have escaped and are often found in mitosis, probably an effort at repair and regeneration.

We are forced to the conclusion that this remarkable injury of the epithelium of the small intestine is responsible for the various abnormal reactions and final lethal intoxication which follow a unit dose of Roentgen radiation over the abdomen of a normal dog. This sensitiveness of the intestinal epithelium to x-rays is not appreciated and should be given proper consideration in clinical work.

In conclusion we wish to express our sincere appreciation for the assistance and advice given us by members of the Department of Roentgenology of the University of California Hospital—Drs. Howard E. Ruggles, Lloyd Bryan, Orrin S. Cook, and Elizabeth Schulze.

BIBLIOGRAPHY.

1. Denis, W., Martin, C. L., and Aldrich, M., *Am. J. Med. Sc.*, 1920, clx, 555.
2. Fromme, G., *Z. Geburtsh. u. Gynäk.*, 1916–17, lxxix, 579.
3. Hall, C. C., and Whipple, G. H., *Am. J. Med. Sc.*, 1919, clvii, 453.
4. McQuarrie, I., and Whipple, G. H., *J. Exp. Med.*, 1922, xxxv, 225.
5. Regaud, C., Nogier, T., and Lacassagne, A., *Arch. électric. méd.*, 1912, xx, 321.

ROENTGEN RAY INTOXICATION.

II. A STUDY OF THE SEQUENCE OF CLINICAL, ANATOMICAL, AND HISTOLOGICAL CHANGES FOLLOWING A UNIT DOSE OF X-RAYS.

BY S. L. WARREN AND G. H. WHIPPLE, M.D.

(From The George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco.)

(Received for publication, August 16, 1921.)

The experimental data given below supplement that of the first paper of this series. All these experiments make it very clear that the initial injury of the epithelium of the small intestine is responsible for the severe clinical reaction and lethal intoxication. The fact that this epithelium is sensitive to the Roentgen rays has not hitherto been clearly recognized. For this reason, it seemed desirable to study carefully this reaction and record the histological and gross changes which follow day by day after the initial exposure.

It is well to recall that the urinary nitrogen in these experiments shows a prompt increase on the day following the exposure (experiments in Paper I and those of Hall and Whipple (2)). This increase is not great and no considerable rise is usually noted until the last day before death. This urinary nitrogen curve does not in any way parallel the clinical reaction, which usually presents a latent period of 24 to 48 hours. It is of great interest to note below that within the first 24 hours, there are distinct histological abnormalities in the epithelium of the small intestine, yet this is always a period free from any clinical evidence of injury but showing a slight rise in urinary nitrogen.

The appearance of clinical intoxication coincides with the actual initial cell disintegration which is so evident in the epithelium of the small intestine in the second 24 hours. It is of interest that the crypt epithelium suffers first and shows evidence of actual necrosis and solution before any change is apparent in the epithelium covering the villi. This may well indicate some undetermined differences in function as well as in histology between these two types of epithelial cell. The epithelium of the villi does not show much disturbance until the 3rd day, when the clinical intoxication is also well marked with bloody diarrhea and bile-stained vomitus.

The severity of the intoxication on the 3rd and 4th days coincides with the disappearance of much of the epithelium all through the small intestine. One may choose to explain this reaction as due to absorption of toxic substances and *invasion of bacteria from the lumen of the injured intestine.* These factors may be in part responsible for the intoxication, but we believe there are other possibilities which must be considered. Some of these are to be reviewed more in detail in subsequent papers of this series.

EXPERIMENTAL OBSERVATIONS.

All materials used in this study are obtained from dogs killed under anesthesia and autopsied at once. Tissues are fixed at once in formaldehyde (10 per cent) and stained usually by the routine hematoxylin-eosin method. Control tissues from normal dogs are fixed and prepared in exactly the same manner. A minimum of mechanical handling before fixation is necessary in this work. Routine examination of all organs in gross and histologically is carefully performed, but in the protocols normal findings are often omitted from the record.

Experiment 1. Lethal Dose of Roentgen Rays over Abdomen. Killed after 2 Hours.

Dog 20–33.—Normal, mongrel, adult, male; weight 32.5 pounds. Dog fasting for 4 days previous to radiation. Roentgen ray exposure over abdomen in four quadrants; no screens used, but usual 2 mm. aluminum filter. In all, 480 milliampere minutes given; E. M. F. of 89 kilovolts; current strength 8 milliamperes; skin-target distance of 10 inches. Exposure given between 3 and 5 p. m. 6 p. m. Dog sacrificed by bleeding under ether anesthesia.

Autopsy.—Performed at once. All viscera examined carefully and found to be normal, except for the kidneys, which show a few scars in the cortex. The intestinal tract shows no congestion and no abnormalities of any sort.

Histological Sections.—All organs and tissues examined in routine sections. There are very few abnormalities. The kidneys show arteriosclerosis and dense scars; the prostate shows some hyperplasia. Spleen and mesenteric glands show a good deal of nuclear fragmentation in centers of follicles.

Gastrointestinal tract at first sight is negative, but careful comparison with sections of normal control dogs convinces one that some of the nuclei in the crypt epithelium of the duodenum and jejunum do show slight abnormalities. These nuclei are fuzzy looking and the chromatin is beginning to show very slight evidence of injury. These changes are much more noticeable in the lymph glands of this region.

Experiment 2. Lethal Dose of Roentgen Rays over Abdomen. Killed after 24 Hours.

Dog 19–130.—Apr. 15, 2 p.m. Normal, old adult, male mongrel; weight 45 pounds. Dog fasting for 3 days previous to radiation. X-ray exposure over abdomen, 450 milliampere minutes; 90 kilovolts; current strength 7.5 milliamperes; spark-gap 9 inches; 2 mm. aluminum filter; skin-target distance 10 inches. Dog given morphia (½ gr.) before exposure.

Apr. 16, 2 p. m. Dog normal in all respects; chloroform anesthesia and exsanguination.

Autopsy.—Done at once. All viscera examined with care and are normal with the following exceptions: *Small intestine* is tightly contracted and contains only bile-stained fluid. Duodenum and jejunum show the mucosa thrown up into longitudinal folds whose crests are specked with ecchymoses. There are a few small scattered ecchymoses all through the small intestine. The mucosa is not markedly congested but is unusually *bile-stained* and this may be due to actual vital staining of the injured cells.

Histological Sections.—Spleen and mesenteric lymph nodes show cell necrosis in lymph follicles and numbers of phagocytes. All of small intestine shows definite nuclear changes in the epithelium of the crypts. Nuclei are pale or show fragmented chromatin material. There is a definite invasion of polymorphonuclear leucocytes. The epithelium of the tips of the villi shows no distinct change as yet.

Experiment 3. Lethal Dose of Roentgen Rays over Abdomen. Killed after 48 Hours.

Dog 19–101.—Apr. 9. Normal, adult, female mongrel; weight 33 pounds. Dog fasting for 4 days and through experiment. X-ray exposure over abdomen, 450 milliampere minutes; 90 kilovolts; current strength 7.5 milliamperes; spark-gap 9 inches; 2 mm. aluminum filter; skin-target distance 10 inches. Dog given morphia (½ gr.) before exposure. Thorax and pelvis screened by lead.

Apr. 10. Dog is normal.

Apr. 11. Dog bright and active, but vomits some bile-stained mucus at 2 p. m. Ether anesthesia and sacrifice at 4 p. m.

Autopsy.—At once. All organs examined with care and are normal except where noted otherwise. Stomach contains a little bile-stained mucus, but mucosa is normal. Small intestine contains a little bile-stained mucus and a little bright red blood in one section; the mucosa in general is pale, but shows scattered ecchymoses. There are longitudinal folds whose crests are pink and dotted with ecchymoses. There are few congested patches in the ileum. Colon is spastic but mucosa normal.

Histological Sections.—Stomach mucosa is normal. There is a sharp line of transition from normal stomach to abnormal duodenal mucosa. Here the duodenal crypts show fragmented nuclei and cell degeneration. A few wandering cells are in evidence. Mitotic figures indicate an effort on the part of the epithelium to regenerate. There is some edema of the submucous tissues. The epithelium covering the villi appears uninjured. This condition is general throughout the small intestine. There is a sharp line of demarcation at the ileocecal valve and the colon mucosa seems practically normal. Colon shows occasional crypts presenting degenerating epithelial cells and invasion by wandering cells. Ovary shows degenerating ova with chromatin fragmentation.

Experiment 4. Lethal Dose of Roentgen Rays over Abdomen. Killed after 3 Days.

Dog 19–50.—Feb. 11. Normal, adult, female spaniel; weight 32 pounds. Dog fasting for 2 days before radiation and through experiment. X-ray exposure over abdomen, 340 milliampere minutes; 97 kilovolts; current strength 7.5 milliamperes; spark-gap 9½ inches; 2 mm. aluminum filter; skin-target distance 10 inches. Thorax and pelvis shielded by lead. Morphia (¼ gr.) before exposure.

Feb. 12. Dog lively; a little frothy vomitus in afternoon.

Feb. 13. Dog less active; blood-stained stools and bile-stained vomitus.

Feb. 14. Dog weak, much bright blood in stools, which contain mucus and clots, very foul smelling; vomiting less frequent. Ether anesthesia and sacrifice.

Autopsy.—At once. Viscera all examined carefully and found normal unless otherwise noted. Kidney: slight scarring of cortex. Pancreas: a few tiny fat necroses close to bile duct. Stomach contains blood-tinged, bile-stained mucus, but its mucosa seems normal. Small intestine contains a little bloody mucus and it is irregularly contracted. *Duodenum* shows a dark, glassy, red mucosa which is thin and in places wrinkled. The mucosa has lost its velvety appearance. This is explained by the histology. The rest of the small intestine shows much the same picture but the surface is mottled and some areas are much more abnormal than others. Peyer's patches are rather conspicuous, due to the thinning of the mucosa. This change in the mucosa begins sharply at the pylorus and ends as sharply at the ileocecal valve. Colon: mucosa is in general pale but there are a few ecchymoses.

Histological Sections.—Unless noted are negative. Lymph follicles in mesenteric glands and spleen show evidence of injury and removal of damaged cells. Ovary shows degenerating and necrotic ova. Bone marrow—lumbar vertebra—shows few marrow cells remaining and much cell debris in large phagocytes. Stomach: some cells in the mucosa appear slightly abnormal but there is nothing definite. *Duodenum:* much of the epithelium covering the villi and lining the crypts has disappeared. In many places, the villi appear as naked polyps, a little swollen and containing dilated capillaries. The crypts are often filled with cell debris and wandering cells. At times mitotic figures are seen in the remaining epithelium. Wandering cells are conspicuous in all this area—usually polymorphonuclears. There is a noticeable edema of the submucosa. The whole structure of the mucosa has collapsed because of the destruction of the epithelium. Some sections show a mucosa which is almost normal. This "escape" of these areas will be discussed later. *Jejunum* and *ileum* show the same extensive destruction of the epithelium with consequent collapse of the familiar structure of the mucosa. Many sections would scarcely be recognized as coming from the small intestine. In places the mucous surface is covered with an exudate of red and white cells with fibrin. Some areas show only a moderate injury, corresponding to the gross appearance.

Experiment 5. Lethal Dose of Roentgen Rays over Abdomen. Killed after 4 Days.

Dog 19–80.—Jan. 21. Adult, female mongrel; weight 20.5 pounds. Dog fasting for 2 days previous to radiation and subsequently. X-ray exposure over abdomen, 350 milliampere minutes; 95 kilovolts; current strength 7.5 milliamperes; spark-gap 9½ inches; skin-target distance 10 inches; 2 mm. aluminum filter. Dog given morphia (¼ gr.) before radiation. Thorax and pelvis screened with lead.

Jan. 22. Dog active and normal.

Jan. 23. Dog less active, but no other clinical symptoms.

Jan. 24. Dog sick with soft stools and usual vomitus.

Jan. 25. Dog prostrated; stools contain fresh blood and clots; temperature is falling and dog becoming comatose; ether anesthesia and sacrifice 90 hours after radiation.

Autopsy.—At once. Unless otherwise stated viscera are normal. Mesenteric glands are enlarged, moist, and congested. Stomach contains blood-tinged mucus and its mucosa is normal. Small intestine contains a little bloody fluid and a few worms. The mucosa in many loops is dark red and glassy, specked with ecchymoses. Other areas are more nearly normal and show the velvety texture of the mucosa. The jejunum seems to be less uniformly injured than the duodenum and ileum. Longitudinal folds with hemorrhagic crests are found in all parts of the small intestine. Colon shows a pale mucosa specked with occasional ecchymoses.

Histological Sections.—The changes noted in this autopsy are almost identical with those described in detail in Experiment 4. The epithelial cells of the mucosa are conspicuous by their absence, and there is more evidence of mitosis and early repair on the part of the few epithelial cells which remain in the injured crypts. There is a moderate grade of edema of the submucosa and some invasion by wandering cells. The congested capillaries in the naked villi are the obvious source of the blood in the intestinal contents. Occasional thrombi are noted in these vessels. Stomach and colon show minimal changes. In similar experiments one may find considerable epithelial degeneration in the crypts of the colon mucosa but it is obvious that this epithelium is much more resistant than is that of the ileum.

DISCUSSION.

We may review the positive features of this Roentgen ray reaction in dogs day by day following the exposure. The first 24 hours show no clinical disturbance. These dogs would pass as normal on the most critical examination. The urinary nitrogen shows a slight but constant increase above normal. The body cells, however, show some definite changes. The spleen and lymph glands show readily recognized cell injury and nuclear fragmentation within 2 hours. The epithelium of the crypts of the small intestine also within 2 hours shows distinct but faint nuclear changes. Within 24 hours, this intestinal epithelium shows nuclear fragmentation and cell injury together with a wandering cell reaction (leucocytes and macrophages). The ova in the ovary show the familiar nuclear reaction of degeneration.

After 2 hours, the small intestine may show a distinct flushing of the mucosa. This may or may not be due wholly to the x-rays, but it may be noted as perhaps similar to the erythema of the skin which may be noted a few hours after an erythema skin dose. The skin may not show distinct injury (burn) for many days after this initial reaction to injury by the x-rays. Apparently the various body cells have individual peculiarities in their reaction to toxic doses of the x-rays, especially as regards the latent period. Compare leucocytes, intestinal epithelium, and skin epithelium.

Within the second 24 hours after a toxic dose of the x-rays, we see many distinct changes from normal. Usually there is definite clinical abnormality, although at times this may be very slight. As a rule, there is loss of appetite and activity. The dog often vomits a little mucus and passes a soft stool or two. Autopsy at this time shows an intestinal tract which may appear normal except for a few ecchymoses in the small intestine. There may be abnormal bile staining of the mucosa, due in part perhaps to the presence of dead epithelium. The intestine in general is apt to be spastic and reacts to the stimuli with very vigorous contractions. This irritation of the muscle coats is probably the result of the early inflammation beginning in the mucosa and submucosa. This spastic tendency, we believe, explains the longitudinal folds which are so conspicuous in the 3rd and 4th days with their crests marked out by congestion and hemorrhage.

Histologically, in the second 24 hours, we find a number of important changes, practically all in the small intestine. The epithelium of the crypts shows severe injury with nuclear destruction and even cell necrosis and desquamation, so that the crypt lumina are filled with cell debris and wandering cells. The epithelium of the villi still remains to all intents normal. The wandering cells are not yet conspicuous in the submucosa and are usually polymorphonuclears. There are a little submucous edema and occasional extravasation of red cells. These changes are conspicuous from the pylorus to the ileocecal valve. The colon shows occasional small patches in which the crypts show similar but less advanced injury.

The third 24 hour period is one of violent clinical disturbance and frank anatomical changes, mostly limited to the small intestine. The dog is constantly retching and frequently vomiting a thick mucoid bile-stained material. There is usually much fluid diarrhea, often with blood and mucus. Tenesmus may be much in evidence. Smears from fecal material show numbers of red and white blood cells, epithelial cells, and bacteria. This period gives marked anatomical changes in a typical case. The entire small intestine from pylorus to ileocecal valve looks raw, red, and thinned out. Some areas may escape and appear relatively normal. We believe these areas of severe injury of the mucosa are to be explained by overlapping areas of exposure. They may also be in the uppermost coils of intestine in the abdomen.

The histological material is of great interest but we cannot review the details. There is almost complete loss of crypt and villous epithelium, and the mucosa collapses into an atypical matrix of villi and empty crypts. Some sections are almost unrecognizable. Villi appear as naked polyps with congested capillaries. A few crypts may contain occasional epithelial cells which are large and contain mitotic figures—an attempt at early repair. The bloody fecal contents are easily explained by this picture. In spite of these raw areas we do not observe the intense inflammatory reaction which might be

expected. There are a little edema and a distinct increase in wandering cells. We believe this point is of much interest as regards the common conception of the intestinal epithelium as the sole barrier between the intestinal bacteria and the blood and lymph stream. This point will be taken up again in detail with a study of its bacteriology.

The fourth 24 hour period is much like the third but shows increasing intoxication. Death usually follows on this day. There is great loss of weight and strength. Vomitus and diarrhea continue unabated and the dog finally goes into coma preceding death. The histology is much like that described for the 3rd day, but evidences of epithelial repair may be more conspicuous and of fascinating interest to the histologist.

It is a common observation that lymph follicles and Peyer's patches show less injury than do the neighboring epithelial cells of the mucosa. Our sections would indicate that the epithelium of the small intestine is at least as sensitive to the x-rays as are the lymphocytes of the same area. In fact, by a large dose of the x-rays, we can actually destroy all of the epithelium, while many clusters of lymphocytes still remain. It is possible, of course, that lymphocytes in different parts of the body have varying susceptibilities to radiation, so that this observation in the intestine may not be applicable to the lymphocytes elsewhere in the body.

It may be noted at this time that a recent publication of Denis, Martin, and Aldrich (1) differs considerably in its interpretation of the abnormalities noted after long x-ray exposures. Rabbits were used and in our experience are not as favorable animals for this work, although the changes are similar. In their discussion, Denis, Martin, and Aldrich suggest that these epithelial changes in the intestine are "the result rather than the cause of the intoxication" and not primarily responsible for the intoxication. We are confident that our experiments give convincing proof that this epithelial injury in the small intestine is due to the x-rays and is in itself responsible for the progressive and lethal intoxication.

SUMMARY.

Roentgen radiation in lethal dosage, given over the abdomen of a normal dog, is followed by a physiological reaction of remarkable uniformity.

The first 24 hour period following the exposure is negative clinically and anatomically, but histologically we see frank changes in the bone marrow, spleen, lymph glands, and ovaries. There are definite nuclear changes with degeneration in the crypt epithelium of the small intestine.

The second 24 hour period shows slight clinical disturbances of gastrointestinal nature (vomitus and diarrhea). The mucosa of the small intestine shows scattered ecchymoses, but the histology of the small intestine is important. The necrosis of the crypt epithelium may be almost complete, while the epithelium of the villi remains practically intact. There are a little edema and invasion of wandering cells.

The third 24 hour period shows increasing clinical disturbance with vomiting and bloody diarrhea. Anatomically the small intestine from the edge of the pylorus to the rim of the ileocecal valve looks raw, red, and inflamed. The crypt and villous epithelium has in large part vanished, leaving a collapsed framework of the mucosa showing a little edema and invasion of wandering cells.

The 4th day marks the peak of the intoxication, and death usually takes place at this time, preceded by coma. Anatomically and histologically the picture is like that of the 3rd day. There is more evidence of mitosis and efforts of repair on the part of the intestinal epithelium.

The stomach is not concerned in this reaction, but the colon may show evidences of a slight injury. The colon is obviously much more resistant than is the small intestine.

We believe the evidence is conclusive that the injury done the epithelium of the small intestine is wholly responsible for the stormy clinical picture and fatal intoxication.

BIBLIOGRAPHY.

1. Denis, W., Martin, C. L., and Aldrich, M., *Am. J. Med. Sc.*, 1920, clx, 555.
2. Hall, C. C., and Whipple, G. H., *Am. J. Med. Sc.*, 1919, clvii, 453.

expected. There are a little edema and a distinct increase in wandering cells. We believe this point is of much interest as regards the common conception of the intestinal epithelium as the sole barrier between the intestinal bacteria and the blood and lymph stream. This point will be taken up again in detail with a study of its bacteriology.

The fourth 24 hour period is much like the third but shows increasing intoxication. Death usually follows on this day. There is great loss of weight and strength. Vomitus and diarrhea continue unabated and the dog finally goes into coma preceding death. The histology is much like that described for the 3rd day, but evidences of epithelial repair may be more conspicuous and of fascinating interest to the histologist.

It is a common observation that lymph follicles and Peyer's patches show less injury than do the neighboring epithelial cells of the mucosa. Our sections would indicate that the epithelium of the small intestine is at least as sensitive to the x-rays as are the lymphocytes of the same area. In fact, by a large dose of the x-rays, we can actually destroy all of the epithelium, while many clusters of lymphocytes still remain. It is possible, of course, that lymphocytes in different parts of the body have varying susceptibilities to radiation, so that this observation in the intestine may not be applicable to the lymphocytes elsewhere in the body.

It may be noted at this time that a recent publication of Denis, Martin, and Aldrich (1) differs considerably in its interpretation of the abnormalities noted after long x-ray exposures. Rabbits were used and in our experience are not as favorable animals for this work, although the changes are similar. In their discussion, Denis, Martin, and Aldrich suggest that these epithelial changes in the intestine are "the result rather than the cause of the intoxication" and not primarily responsible for the intoxication. We are confident that our experiments give convincing proof that this epithelial injury in the small intestine is due to the x-rays and is in itself responsible for the progressive and lethal intoxication.

SUMMARY.

Roentgen radiation in lethal dosage, given over the abdomen of a normal dog, is followed by a physiological reaction of remarkable uniformity.

The first 24 hour period following the exposure is negative clinically and anatomically, but histologically we see frank changes in the bone marrow, spleen, lymph glands, and ovaries. There are definite nuclear changes with degeneration in the crypt epithelium of the small intestine.

The second 24 hour period shows slight clinical disturbances of gastrointestinal nature (vomitus and diarrhea). The mucosa of the small intestine shows scattered ecchymoses, but the histology of the small intestine is important. The necrosis of the crypt epithelium may be almost complete, while the epithelium of the villi remains practically intact. There are a little edema and invasion of wandering cells.

The third 24 hour period shows increasing clinical disturbance with vomiting and bloody diarrhea. Anatomically the small intestine from the edge of the pylorus to the rim of the ileocecal valve looks raw, red, and inflamed. The crypt and villous epithelium has in large part vanished, leaving a collapsed framework of the mucosa showing a little edema and invasion of wandering cells.

The 4th day marks the peak of the intoxication, and death usually takes place at this time, preceded by coma. Anatomically and histologically the picture is like that of the 3rd day. There is more evidence of mitosis and efforts of repair on the part of the intestinal epithelium.

The stomach is not concerned in this reaction, but the colon may show evidences of a slight injury. The colon is obviously much more resistant than is the small intestine.

We believe the evidence is conclusive that the injury done the epithelium of the small intestine is wholly responsible for the stormy clinical picture and fatal intoxication.

BIBLIOGRAPHY.

1. Denis, W., Martin, C. L., and Aldrich, M., *Am. J. Med. Sc.*, 1920, clx, 555.
2. Hall, C. C., and Whipple, G. H., *Am. J. Med. Sc.*, 1919, clvii, 453.

expected. There are a little edema and a distinct increase in wandering cells. We believe this point is of much interest as regards the common conception of the intestinal epithelium as the sole barrier between the intestinal bacteria and the blood and lymph stream. This point will be taken up again in detail with a study of its bacteriology.

The fourth 24 hour period is much like the third but shows increasing intoxication. Death usually follows on this day. There is great loss of weight and strength. Vomitus and diarrhea continue unabated and the dog finally goes into coma preceding death. The histology is much like that described for the 3rd day, but evidences of epithelial repair may be more conspicuous and of fascinating interest to the histologist.

It is a common observation that lymph follicles and Peyer's patches show less injury than do the neighboring epithelial cells of the mucosa. Our sections would indicate that the epithelium of the small intestine is at least as sensitive to the x-rays as are the lymphocytes of the same area. In fact, by a large dose of the x-rays, we can actually destroy all of the epithelium, while many clusters of lymphocytes still remain. It is possible, of course, that lymphocytes in different parts of the body have varying susceptibilities to radiation, so that this observation in the intestine may not be applicable to the lymphocytes elsewhere in the body.

It may be noted at this time that a recent publication of Denis, Martin, and Aldrich (1) differs considerably in its interpretation of the abnormalities noted after long x-ray exposures. Rabbits were used and in our experience are not as favorable animals for this work, although the changes are similar. In their discussion, Denis, Martin, and Aldrich suggest that these epithelial changes in the intestine are "the result rather than the cause of the intoxication" and not primarily responsible for the intoxication. We are confident that our experiments give convincing proof that this epithelial injury in the small intestine is due to the x-rays and is in itself responsible for the progressive and lethal intoxication.

SUMMARY.

Roentgen radiation in lethal dosage, given over the abdomen of a normal dog, is followed by a physiological reaction of remarkable uniformity.

The first 24 hour period following the exposure is negative clinically and anatomically, but histologically we see frank changes in the bone marrow, spleen, lymph glands, and ovaries. There are definite nuclear changes with degeneration in the crypt epithelium of the small intestine.

The second 24 hour period shows slight clinical disturbances of gastrointestinal nature (vomitus and diarrhea). The mucosa of the small intestine shows scattered ecchymoses, but the histology of the small intestine is important. The necrosis of the crypt epithelium may be almost complete, while the epithelium of the villi remains practically intact. There are a little edema and invasion of wandering cells.

The third 24 hour period shows increasing clinical disturbance with vomiting and bloody diarrhea. Anatomically the small intestine from the edge of the pylorus to the rim of the ileocecal valve looks raw, red, and inflamed. The crypt and villous epithelium has in large part vanished, leaving a collapsed framework of the mucosa showing a little edema and invasion of wandering cells.

The 4th day marks the peak of the intoxication, and death usually takes place at this time, preceded by coma. Anatomically and histologically the picture is like that of the 3rd day. There is more evidence of mitosis and efforts of repair on the part of the intestinal epithelium.

The stomach is not concerned in this reaction, but the colon may show evidences of a slight injury. The colon is obviously much more resistant than is the small intestine.

We believe the evidence is conclusive that the injury done the epithelium of the small intestine is wholly responsible for the stormy clinical picture and fatal intoxication.

BIBLIOGRAPHY.

1. Denis, W., Martin, C. L., and Aldrich, M., *Am. J. Med. Sc.*, 1920, clx, 555.
2. Hall, C. C., and Whipple, G. H., *Am. J. Med. Sc.*, 1919, clvii, 453.

expected. There are a little edema and a distinct increase in wandering cells. We believe this point is of much interest as regards the common conception of the intestinal epithelium as the sole barrier between the intestinal bacteria and the blood and lymph stream. This point will be taken up again in detail with a study of its bacteriology.

The fourth 24 hour period is much like the third but shows increasing intoxication. Death usually follows on this day. There is great loss of weight and strength. Vomitus and diarrhea continue unabated and the dog finally goes into coma preceding death. The histology is much like that described for the 3rd day, but evidences of epithelial repair may be more conspicuous and of fascinating interest to the histologist.

It is a common observation that lymph follicles and Peyer's patches show less injury than do the neighboring epithelial cells of the mucosa. Our sections would indicate that the epithelium of the small intestine is at least as sensitive to the x-rays as are the lymphocytes of the same area. In fact, by a large dose of the x-rays, we can actually destroy all of the epithelium, while many clusters of lymphocytes still remain. It is possible, of course, that lymphocytes in different parts of the body have varying susceptibilities to radiation, so that this observation in the intestine may not be applicable to the lymphocytes elsewhere in the body.

It may be noted at this time that a recent publication of Denis, Martin, and Aldrich (1) differs considerably in its interpretation of the abnormalities noted after long x-ray exposures. Rabbits were used and in our experience are not as favorable animals for this work, although the changes are similar. In their discussion, Denis, Martin, and Aldrich suggest that these epithelial changes in the intestine are "the result rather than the cause of the intoxication" and not primarily responsible for the intoxication. We are confident that our experiments give convincing proof that this epithelial injury in the small intestine is due to the x-rays and is in itself responsible for the progressive and lethal intoxication.

SUMMARY.

Roentgen radiation in lethal dosage, given over the abdomen of a normal dog, is followed by a physiological reaction of remarkable uniformity.

The first 24 hour period following the exposure is negative clinically and anatomically, but histologically we see frank changes in the bone marrow, spleen, lymph glands, and ovaries. There are definite nuclear changes with degeneration in the crypt epithelium of the small intestine.

The second 24 hour period shows slight clinical disturbances of gastrointestinal nature (vomitus and diarrhea). The mucosa of the small intestine shows scattered ecchymoses, but the histology of the small intestine is important. The necrosis of the crypt epithelium may be almost complete, while the epithelium of the villi remains practically intact. There are a little edema and invasion of wandering cells.

The third 24 hour period shows increasing clinical disturbance with vomiting and bloody diarrhea. Anatomically the small intestine from the edge of the pylorus to the rim of the ileocecal valve looks raw, red, and inflamed. The crypt and villous epithelium has in large part vanished, leaving a collapsed framework of the mucosa showing a little edema and invasion of wandering cells.

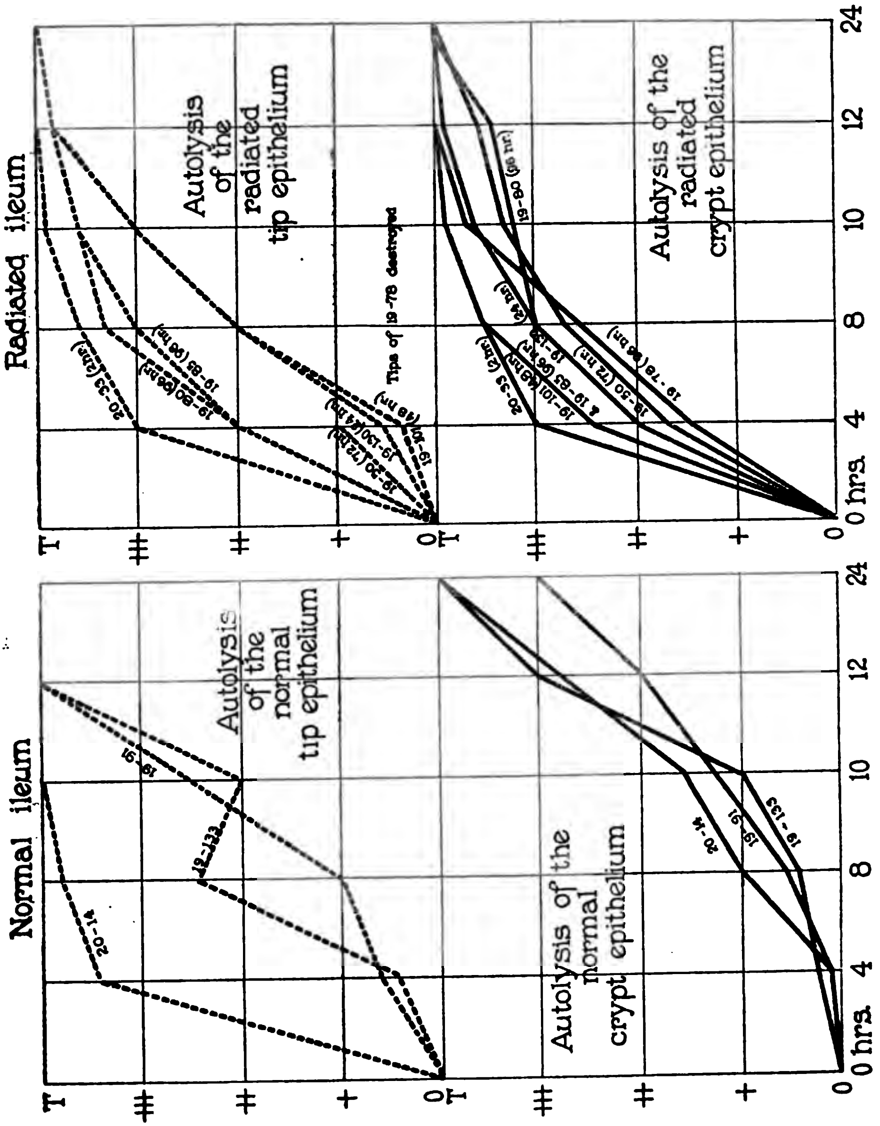
The 4th day marks the peak of the intoxication, and death usually takes place at this time, preceded by coma. Anatomically and histologically the picture is like that of the 3rd day. There is more evidence of mitosis and efforts of repair on the part of the intestinal epithelium.

The stomach is not concerned in this reaction, but the colon may show evidences of a slight injury. The colon is obviously much more resistant than is the small intestine.

We believe the evidence is conclusive that the injury done the epithelium of the small intestine is wholly responsible for the stormy clinical picture and fatal intoxication.

BIBLIOGRAPHY.

1. Denis, W., Martin, C. L., and Aldrich, M., *Am. J. Med. Sc.*, 1920, clx, 555.
2. Hall, C. C., and Whipple, G. H., *Am. J. Med. Sc.*, 1919, clvii, 453.



TEXT-FIG. 2. Comparison of the autolysis of the ileum of normal and of radiated dogs.

TABLE I.

Autolysis during Periods of Hours as Indicated.

	Control.					2 hrs. after x-rays.					24 hrs. after x-rays.					48 hrs. after x-rays.					72 hrs. after x-rays.				
Hrs. autolysis.	4	8	10	12	24	4	8	10	12	24	4	8	10	12	24	4	8	10	12	24	4	8	10	12	24
Duodenum crypt	0	0	A	A	C	A	C	C	D	—	B	C	D	D	—	B	C	D	D	—	B	C	D	D	—
“ villi	A	A	B	C	D	“	B	“	C	D	“	“	C	“	—	“	“	C	“	—	A	B	C	“	—
Jejunum crypt	0	0	A	B	C	B	C	D	D	—	“	“	D	“	—	“	“	D	“	—	B	C	“	“	D
“ villi	0	A	B	C	D	0	A	B	C	D	A	“	C	“	—	A	B	C	“	D	—	—	—	C	—
Ileum crypt	0	“	A	B	C	C	C	D	D	—	B	“	D	“	—	B	C	D	“	—	B	C	D	D	—
“ villi	A	B	B	C	D	B	“	“	“	—	A	B	C	“	—	A	B	C	“	—	A	—	—	—	—
Colon	0	A	“	“	“	A	B	C	“	—	B	C	“	“	—	“	C	“	“	—	“	B	C	D	D
Stomach	A	“	“	“	“	B	C	D	“	—	A	B	“	“	—	“	B	“	“	D	“	“	“	“	“
Liver	“	B	“	“	“	“	“	C	“	—	“	C	“	“	—	“	“	“	“	—	“	“	“	“	“
Pancreas	0	A	“	“	“	“	“	“	“	—	B	“	“	“	—	“	“	“	C	D	“	“	A	B	“
Spleen	A	B	“	“	“	“	“	“	“	—	C	“	D	“	—	B	C	“	D	“	“	“	B	“	“
Kidney	0	A	“	“	C	A	B	B	C	C	0	A	A	B	C	A	A	B	C	C	B	“	C	C	“

Degree of autolysis indicated as follows (see complete description under Histological method): 0 means zero autolysis. A means slight autolysis. B means well advanced autolysis. C means marked autolysis. D means total autolysis.

tubules and the glomeruli, mainly because of their difference in autolysis; in the liver, the parenchyma cells are contrasted with the bile ducts.

In the normal intestine, the autolytic digestion of the mucosa starts in the tips of the villi and progresses fairly slowly and evenly to the bases of the crypts. Even at the end of 24 hours, the crypt epithelium may be fairly well preserved while the tip epithelium is almost totally autolyzed. In the *radiated animal*, on the other hand, *the crypt epithelium autolyzes at a greater speed than the tip epithelium* and is practically completely autolyzed in 10 to 12 hours or earlier, while even after 12 hours of autolysis the tip epithelium in the same animal may be fairly well preserved (compare Text-figs. 1 and 2).

Discussion of Experiments.

Normal Autolysis.—Three normal dogs are sacrificed under anesthesia. Sections of abdominal viscera are autolyzed at body temperature in 0.9 per cent sodium chloride solution saturated while warm with chloroform as described above. The usual course of digestion is from without inward and the progress is steady and continuous. The digestion in the gastrointestinal sections progresses from the villi to the bases of the crypts. Comparisons are made of the penetration of the autolysis as well as of the progress of cell digestion.

Stomach: The chief and parietal cells of the gastric mucosa are more susceptible to autolysis than are the tip cells. The former are autolyzed rapidly and are totally digested at the end of 10 hours. The tip cells autolyze somewhat more slowly and are completely digested in 24 hours.

Duodenum, jejunum, ileum, and *colon* are quite similar in the type of their autolysis. The tip epithelium is the first to autolyze. In these control experiments the time may vary from 10 to 24 hours before completion of the digestion. Considerable variation is to be noted in this reaction of the epithelium of the villi. The autolysis of the crypts is usually delayed 4 to 8 hours, after which it proceeds regularly though usually not to completion in 24 hours (Text-figs. 1 and 2).

Spleen pulp is found to autolyze 4 to 8 hours faster than the Malpighian bodies, which, as a rule, are not completely autolyzed by 24 hours. Their centers are fairly well preserved at 12 hours. The pulp at this time is usually pretty well digested.

Pancreas digests pretty well as a unit. The islands of Langerhans autolyze on the average 2 to 4 hours more slowly at first than the parenchyma but later the speed varies considerably so that they may totally digest in 10 hours, or only partly digest in the full 24 hours.

Kidney convoluted tubules are very sensitive to autolysis. Usually they are completely digested in 8 or 10 hours. The remainder of the *kidney parenchyma* consisting of the straight and collecting tubules and the glomeruli is more slowly digested, the full 24 hours usually being required for their complete autolysis.

Liver parenchyma and bile capillaries autolyze as a unit.

Autolysis of Radiated Organs.—Seven normal dogs are exposed to large amounts of radiation, given diffusely over the abdomen, and sacrificed at definite periods after exposure. Autolysis specimens are studied (as above) in regard to the progress of digestion in the specialized cells of the various organs.

Stomach epithelium, though the speed of autolysis may seem to have increased slightly, is digested well within normal limits. This is true for the whole series of dogs which were killed 2, 24, 48, 72, and 96 hours after radiation.

Intestinal epithelium shows a striking change from the controls. The speed of autolysis of the tip epithelium in each case is practically within normal limits. The great change has occurred in the speed of the autolysis of the crypt epithelium. The curves in Text-figs. 1 and 2 illustrate this very well.

The speed of autolysis of the crypt epithelium is rapid and must be due to the immediate effect of radiation upon these cells. The most pronounced increase in the speed of autolysis occurs within the first 2 hours after exposure to radiation. The change produced in these cells, or what is left of them, is permanent up to the time of the death of the animal 4 days later. Even after a lapse of 4 days, these cells autolyze faster than the normal crypt epithelium. These curves correspond remarkably well, indicating that the increase in autolysis is due to a change in the cell protoplasm which persists at least for 4 days after radiation. In all of the animals exposed to radiation, the crypt epithelium of the small intestine is completely autolyzed in 12 hours in the duodenum, ileum, and jejunum, while in the experiments with normal animals, the crypt epithelium is rarely autolyzed completely in 24 hours and usually the autolysis is only well advanced by that time.

Colon shows an increase in the speed of autolysis of the crypt epithelium and possibly of the tip epithelium, though the latter is less pronounced. The effect of the radiation seems to decrease or wear off slightly, for the speed of autolysis is somewhat slower and approaches the normal in the 3rd and 4th days after exposure to radiation. This may be connected with the fact that there is also relatively little injury to the colon, anatomically or histologically. The most pronounced increase in the speed of autolysis in the colon epithelium occurs, therefore, within the first 48 hours.

Spleen shows an apparent increase in the speed of autolysis, especially in the first 24 hours after exposure to the x-rays. The 72 hour curve is within normal limits, while both the 48 and the 96 hour curves show an increased speed in autolysis.

Pancreas shows a slight but definite increase in the speed of autolysis, especially during the first 48 hours after exposure. The 96 hour curves seem to indicate also that the speed of autolysis is increased, but the 72 hour curves are irregular as in the spleen series. The islands of Langerhans autolyze as a rule somewhat faster than the pancreatic parenchyma.

Kidney does not show changes in the speed of autolysis after exposure to radiation. The convoluted tubules are totally autolyzed in 8 hours. The other tubules and the glomeruli, while autolyzing more slowly, apparently digest at the normal rate. This would suggest that radiation has little effect upon the kidney.

Liver parenchyma shows a definite increase in the speed of autolysis, with a maximum in the specimens removed 2 hours after the radiation. The 24 and 48 hour experiments show less increase above normal.

DISCUSSION.

Many observers have studied ferment changes *in vitro* in normal organs or tumor tissues exposed to x-rays before the unit period of autolysis. Much of this work has been reviewed by Hall and Whipple and need not be discussed. Our experiments indicate that the spleen, liver, and pancreas taken from a radiated animal will show a more rapid autolysis than similar tissues from control normal animals under similar conditions of autolysis. The kidney shows no such change by the methods used. We may recall that the spleen shows obvious histological injury after exposure to the x-rays, but such changes are lacking in the liver, pancreas, and kidney.

The stomach and colon are much more resistant than the small intestine, but we shall publish experiments to show that large and permanent gastric and colon ulcers may be produced by the x-rays.

The *duration of the injury* done the body cells is of much interest to us. We note that abnormal autolysis of the spleen, liver, and pancreas is most striking within 24 hours after the radiation. After 48 hours, there is a distinct decrease in this reaction as observed in dogs killed 72 and 96 hours following the lethal dose of x-rays. In the small intestine, it is easy to demonstrate the abnormal autolysis in dogs even 96 hours after the x-ray exposure. This indicates in a sense that these cells are suffering from a persistence of the injury exerted by the x-rays, even during the 4th day after the exposure. It is significant that the clinical symptoms always reach a crisis on the 4th day. If this period is survived, the dog usually goes on to recovery.

We may note in passing that the cell necrosis and injury in the intestine as in the skin is associated with a minimal wandering cell reaction. This point deserves further study. We shall record also the fact that ulcers caused by the x-rays are as stubborn in healing in the stomach and intestine as is so well recognized in the skin.

SUMMARY.

Exposure to large doses of x-rays will cause notable increase in the speed of autolysis of the crypt or secretory epithelium of the dog's small intestine. These changes can be demonstrated readily in

material obtained from dogs killed 2, 24, 48, 72, or 96 hours after the initial radiation (Text-figs. 1 and 2).

In the radiated dogs the secretory crypt epithelium of the small intestine autolyzes first and the epithelium of the villi last, while the reverse is true in the normal control small intestine. These abnormalities of autolysis associated with lethal Roentgen ray exposures can be demonstrated for the small intestine over the whole 4 day period subsequent to radiation.

The colon shows little change and the stomach no demonstrable changes in autolysis under like conditions. The kidney likewise is negative.

The spleen, lymph glands, liver, and pancreas show a moderate increase in speed of autolysis in tissues taken from radiated animals within 48 hours of the initial exposure.

What the significance of this disturbance of cell ferments in the intestinal mucosa may be, we cannot pretend to say. At least these observations strengthen one's confidence in the profound functional disturbance of this important intestinal epithelium—a disturbance which we believe is responsible for the clinical abnormalities and fatal intoxication.

BIBLIOGRAPHY.

1. Hall, C. C., and Whipple, G. H., *Am. J. Med. Sc.*, 1919, clvii, 453.

A STUDY OF RENAL FUNCTION IN ROENTGEN RAY INTOXICATION.

Resistance of Renal Epithelium to Direct Radiation.

By IRVINE McQUARRIE, M.D., and G. H. WHIPPLE, M.D.

(From The George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco.)

(Received for publication, August 17, 1921.)

In an earlier publication (Hall and Whipple (4)), the noticeable clinical similarity between intestinal obstruction and systemic x-ray intoxication has been pointed out. We have recorded elsewhere (10, 11) an interesting transient disturbance of renal function in the intoxication due to intestinal obstruction and in certain proteose intoxications. This disturbance of renal function is considerable and plays an important part in certain of the clinical reactions (intestinal obstructions). We believe this is the first instance of a well established functional depression of renal secretory function totally unassociated with any abnormalities of renal structure. Because of this peculiar disturbance of renal function in certain proteose intoxications, we took up a study of renal function in systemic x-ray intoxication with the keenest interest. We may say at once that our experiments show a totally different picture, with the renal function uninvolved in the toxic disturbance of general x-ray intoxication. There is a minimal disturbance of renal function by the direct action of very large doses of the hard x-rays but no anatomical evidence of cell injury. We feel confident that the clinician may use the x-rays over the kidney areas with confidence that renal tissue is resistant to the hard Roentgen rays. Our experiments indicate that the kidney epithelium is much more resistant to x-ray injury than is the epithelium of the small intestine.

Inspection of the literature shows that work has been done to determine possible injury of the kidney by the x-rays. However, most of the reports deal with the morphological changes produced or consist of clinical observations made by use of the older, less reliable means of determining the presence of functional disturbances. Warthin (18) noted pathological changes in the kidneys of several leucemia patients who had received x-ray treatment before death and reports finding definite changes in the tubular epithelium following prolonged percutaneous irradiation of the kidneys of white mice and rats. Rosenstern (13), Linser and Helber (9), Schleip and Hildebrand (15), von Jaksch (6), Heymann (5), Přibram and Rotky (12), and others likewise claim to have found evidence of an x-ray nephritis. It has been thought by many that the inability of injured kidneys to do their work efficiently must be considered as an essential factor in the constitutional x-ray reaction. While Edsall and Pemberton (3) considered the primary intoxication to be quite independent of any direct effect of the x-rays upon the kidneys, they suggest that the kidneys may become overtaxed by the increased work thrown upon them to such an extent that their failure may secondarily intensify the symptoms of intoxication.

There are many workers, however, who have failed to find any definite clinical or postmortem evidence of an x-ray nephritis. Among those who have reported negative findings may be mentioned Krause and Ziegler (8) and Buschke and Schmidt (2). The latter workers incised the abdominal wall and exposed the kidneys of a number of rats directly to large doses of the x-rays. The animals died a few weeks later, two of them only showing albumin in the urine and pathological changes in the structure of the kidneys at autopsy. Since in these two cases the changes might well have been due to some complication following the operations, these workers concluded that the renal epithelium is very resistant to the x-rays and that consequently there is no such thing as an x-ray nephritis. Warthin, whose work is more recent and freer from complications, admits that the anatomical changes were so slight in his experimental animals that they might be overlooked unless special histological technique is employed. Krause (7) in 300 cases finds no albumin or leucocytes in the urine after radiation. Stephan (16) in a small series of cases claims to cure anuria and produce a diuresis in 24 hours by means of ⅙ to ¼ of an erythema dose over the kidneys. This brief reference to the work previously done on the subject suffices to show the decided lack of agreement among those who have given any attention to it. There is obvious need for a thorough functional study of the problem with employment of the comparatively accurate methods now available for measuring the functional activity of the kidneys under conditions of carefully controlled x-ray administration.

Method.

Normal adult dogs were used exclusively in all of the experiments. The animals were selected after being under observation for a period of several days during which time the functional tests to be used in the subsequent experiments were performed on them. The data recorded in this preliminary examination on each dog serve as a standard with which to compare results obtained in subsequent experiments. At the end of this fore period the animal was exposed to a carefully measured dose of x-rays, following which the renal function was determined daily for a period of a week or more.

The hard Roentgen rays of the Coolidge tube were used exclusively, the softer rays being filtered out with 2 mm. aluminum filters. In a very few cases only did we have leakage with slight tendency to pigmentation and denudation of the skin. All exposures were percutaneous and the distance from the target (anticathode) to the surface of the animal's body measured in all cases 25 cm. The ammeter was made to read at approximately 8 milliamperes in most of the experiments, although currents as high as 10 milliamperes and as low as 5 milliamperes were employed in a few instances. Our work confirms the observation of Hall and Whipple (4) that in dogs the toxicity of the hard rays increases with the increase in spark-gap. Our experience has shown that a 9 inch spark-gap is a convenient one for producing the desired experimental intoxication and that this required an E. M. F. of about 90 kilovolts. With a few exceptions, this voltage was employed.

In a part of the experiments moderate sized sublethal doses of x-rays were given at intervals over a considerable period of time, a short rest on a mixed diet being given between exposures. In the remainder, huge and frequently fatal doses were given singly or in succession. It was considered that between these extremes no impairment of function, whether prompt or delayed in its appearance, could escape detection. In order to understand the nature or the mechanism of any disturbance which might be observed, different regions of the body were exposed separately in the various experiments.

For a period of 1 to 3 days preceding and 5 to 6 days subsequent to the radiation, the dogs were given only water. During resting intervals, the dogs received the usual kennel mixed diet of table scraps, cooked meat, bread, rice, bones, and so forth.

The methods chosen for the measurement of the renal function were those employed by us in studies on the renal function in intestinal obstruction (10); namely, the phenolsulfonephthalein elimination test (14) and that of determining the capacity of the kidneys to excrete urea. The former method was carried out according to the procedure outlined by its authors, the Duboscq colorimeter being used for the determination of the dye. For the latter we made a daily determination of the blood urea in all experiments and determined in many cases the ratio

$$\frac{\text{Urea in 1 hr.'s urine}}{\text{Urea per 100 cc. of blood}}$$

after the intravenous injection of comparatively large amounts of urea in saline solution. A decrease in this ratio indicates a definite impairment of kidney function as shown by Addis and Watanabe (1). The tests were made after the exposure to the x-rays exactly as in the fore period except that in cases of extreme intoxication with resulting loss of fluid from the body, more fluid was given intravenously in order that the loss might be made up as far as possible. The urea of both the blood and the urine was determined by the Van Slyke-Cullen modification of the Marshall urease method (17). Morphine sulfate ($\frac{1}{8}$ to $\frac{1}{4}$ gr. doses) was administered subcutaneously 1 hour before the animal was placed upon the x-ray table to insure quiet during the period of exposure.

EXPERIMENTAL DATA.

The experimental data assembled below consist of three types of experiments. The first includes a group of observations on several different animals in which *sublethal doses of x-rays* were given at intervals over relatively long periods of time. In these experiments, the thorax and the abdomen, including the kidneys, were alternately exposed. The purpose of this series was to ascertain whether or not repeated x-ray treatment over a long period of time is capable of producing any gradual changes in the renal function. Many have feared the possibility of cumulative action resulting in permanent damage to the kidneys.

Dog 19–85 (*Tables I and II*). *Autopsy Record.*—Dog sacrificed under ether anesthesia 4 days after the last radiation, Apr. 5. The kidneys in gross are quite normal. Bladder normal. Histological sections show normal stroma, vessels, and glomeruli. There are no scars. Convoluted tubules are normal.

TABLE I.

Large Sublethal Doses of X-Rays Given over Abdomen and Thorax. Renal Function Unimpaired.

Dog 19-85. Adult female spitz.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
Nov. 30	24.9	23	75	Normal.
Nov. 30	190 milliampere minutes, x-rays over abdomen and kidneys; 90 kilovolts; 10 milliamperes.			
Dec. 1	24.7	22	71	Normal.
" 3	24.2	36	74	"
" 5	23.7	34	54	Slight diarrhea.
" 6	23.2	36	55	No diarrhea. Normal.
" 7	22.7	40	63	Normal.
Dec. 16	220 milliampere minutes, x-rays over thorax and neck; 90 kilovolts; 10 milliamperes.			
Dec. 17	24.3	32	62	Normal.
" 18	23.9	30	69	—
" 19	23.1	42	57	—
" 20	23.0	31	52	—
" 21	22.9	30	67	Normal.
Dec. 31	350 milliampere minutes, x-rays over abdomen and back; 90 kilovolts; 8 milliamperes.			
Jan. 1	22.6	—	67	Normal.
" 2	22.0	22	67	—
" 3	21.8	20	71	—
" 4	21.2	23	63	Normal.
" 5	20.8	30	67	—
" 6	20.4	28	63	—
Feb. 4	300 milliampere minutes, x-rays over abdomen and back; 95 kilovolts; 8 milliamperes.			
Feb. 8	23.0	34	63	Normal.
" 9	23.0	30	63	—
" 10	22.6	28	66	—
" 11	22.4	24	64	—
" 12	22.2	25	63	—

Mixed diet in intervals between experiments; no food for 5 days following exposures.

It will be seen from Tables I to IV that the experiments of this group are a unit in showing that the excretory function of the kidneys, as measured by their ability to eliminate a foreign substance such as phenolsulfonephthalein or the normal urinary constituent, urea, is not disturbed by this procedure. It is evident that with the doses given there is no sign of any cumulative effect being produced, whether the thorax or abdomen or both be exposed.

TABLE II.

Large Sublethal Doses of X-Rays Given over Abdomen and Thorax. Renal Function Unimpaired.

Dog 19–85. Adult female spitz.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
Dec. 21	9.00–10.00	—	—	30	—	—	Normal.
	10.00–12.00	20	3.59	262	13.7	67	—
Mar. 4	350 milliampere minutes, x-rays over thorax; 95 kilovolts; 5 milliamperes.						
Mar. 7	9.00–11.00	—	—	28	—	63	Normal.
" 8	—	—	—	27	—	64	—
" 9	10.00–12.00	20	3.78	270	14.0	67	Normal.
Apr. 1	350 milliampere minutes, x-rays over abdomen; 105 kilovolts; 5 milliamperes.						
Apr. 4	12.00–2.00	—	—	39	—	65	Moderate intoxication.
" 5	3.00–5.00	20	3.42	278	12.3	—	Same condition.

The second type of experiment is represented by the observations made on Dogs 19–127 and 19–140 (Tables V to VIII) in which a lethal dose of the x-rays was given over the abdomen with the kidneys and the remainder of the body protected from the direct rays by the leaden sheets. This type of experiment was designed to demonstrate whether or not a poisonous substance capable of injuring the kidneys is produced in the irradiated gastrointestinal tract and carried by the blood stream to the kidneys. Reference to the tables shows that the function of the kidneys remains entirely normal after the exposure,

TABLE III.

Repeated Sublethal Doses of X-Rays over Thorax and Abdomen. Renal Function Unchanged.

Dog 19-78. Adult female Airedale. (Autopsy, see Table XV.)

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
Nov. 23	28.5	30	68	Normal.
Nov. 25	190 milliampere minutes, x-rays over thorax and neck; 85 kilovolts; 5 milliamperes.			
Nov. 26	28.1	18	63	Normal.
" 27	27.5	22	57	—
" 28	27.4	20	60	—
" 29	27.1	—	58	—
" 30	26.5	33	58	—
Dec. 1	26.4	28	60	—
" 2	26.4	23	68	No food since exposure.
" 5	26.0	20	60	Mixed diet.
" 10	27.5	—	62	—
Dec. 10	190 milliampere minutes, x-rays over abdomen and back; 90 kilovolts; 5 milliamperes.			
Dec. 11	27.5	40	59	Normal.
" 12	27.0	40	59	—
" 13	26.2	40	54	—
" 14	26.0	41	59	—
" 15	25.7	36	54	—
" 16	24.9	22	59	—
Dec. 27	240 milliampere minutes, x-rays over thorax; 90 kilovolts; 5 milliamperes.			
Dec. 28	26.6	36	64	Normal.
" 29	26.5	47	64	—
" 30	26.3	50	62	—
" 31	25.5	43	63	—
Jan. 1	25.0	33	59	—

Dog not fed for day or two before exposure and for 5 days after each radiation.

although the dose of x-rays produced profound intoxication and death. The evidence against any indirect action of the x-rays upon the kidney function or upon the structure of the kidney is very clear-cut. The blood urea showed a definite increase in both cases, but this was due to accelerated tissue destruction rather than to retention, as is shown by the fact that the urea ratio (Tables VI and VIII) remained normal even when additional urea was injected intravenously. The results indicate the absence of any hypothetical nephrotoxic substance in the blood during the period of intoxication, and thus point out a

TABLE IV.

Repeated Increasing Doses of X-Rays over Thorax and Abdomen. Renal Function Undisturbed.

Dog 19–50. Young adult female. (Autopsy, see Table XVI.)

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
Dec. 5	30.40	28	74	—
Dec. 5	210 milliampere minutes, x-rays over thorax; 90 kilovolts; 10 milliamperes.			
Dec. 6	29.50	22	72	Normal.
" 7	29.30	31	66	—
" 8	28.20	31	63	—
" 9	27.00	34	55	—
" 10	26.40	34	63	—
" 12	26.50	—	—	—
" 14	26.50	28	70	—
" 15	27.10	33	—	—
Dec. 16	210 milliampere minutes, x-rays over abdomen and back; 90 kilovolts; 10 milliamperes.			
Dec. 17	27.15	37	56	Normal.
" 18	27.00	31	68	—
" 19	26.70	34	65	—
" 20	25.40	30	63	—
Dec. 31	350 milliampere minutes, x-rays over thorax; 90 kilovolts; 7.5 milliamperes.			
Jan. 1	26.70	—	72	Normal.
" 2	26.50	28	67	—
" 3	26.00	26	61	—
" 4	25.50	22	61	—
" 5	25.40	24	63	—
" 6	25.30	20	66	—
" 7	25.00	21	63	—

TABLE V.

Lethal Dose of X-Rays over Abdomen with Kidneys Shielded. Renal Function Unchanged.

Dog 19–127. Adult male mongrel.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
June 9	28.4	32	74	
" 10	28.3	30	72	Normal.
June 10	504 milliampere minutes, x-rays over abdomen, kidneys completely shielded; 90 kilovolts; 8 milliamperes.			
June 11	27.8	27	76	Normal.
" 12	26.3	35	67	Quiet.
" 13	26.1	44	68	Dull and weak.
" 14	25.5	46	66	Intoxication increasing.
" 15	—	53	59	Grave intoxication and collapse. Death in night.

TABLE VI.

Lethal Dose of X-Rays over Abdomen with Kidneys Shielded. Renal Function Unchanged.

Dog 19–127. Adult male mongrel.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
June 9	8.50– 9.50	—	0.160	32	5.0	—	—
	10.00–11.00	20	2.382	240	9.9	74	Normal.
	11.00–12.00	—	1.856	201	9.2	—	—
June 10	504 milliampere minutes, x-rays over abdomen, kidneys shielded; 90 kilovolts; 8 milliamperes.						
June 14	8.45– 9.45	—	0.144	46	3.1	—	—
	10.00–11.00	20	2.215	237	9.3	66	Severe intoxication.
	11.00–12.00	—	1.693	192	8.8	—	—

TABLE VII.

Lethal Dose of X-Rays over Abdomen with Kidneys Shielded.
Renal Function Unchanged.

Dog 19–140. Young adult female collie.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
July 7	21.7	34	70	—
" 8	20.5	32	72	Normal.
July 8	450 milliampere minutes, x-rays over abdomen, kidneys shielded; 90 kilovolts; 8 milliamperes.			
July 9	19.7	30	70	Normal.
" 10	19.3	34	63	Dull and weak.
" 11	19.0	43	63	Severe intoxication; bloody diarrhea; death in night.

TABLE VIII.

Lethal Dose of X-Rays over Abdomen with Kidneys Shielded.
Renal Function Unchanged.

Dog 19–140. Young adult female collie.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
July 8	7.55– 8.55	—	0.160	32	5.0	—	—
	9.00–10.00	20	3.028	244	12.4	72	Normal.
	10.00–11.00	—	2.353	219	10.7	—	—
July 8	450 milliampere minutes, x-rays over abdomen, kidneys shielded; 90 kilovolts; 8 milliamperes.						
July 11	8.30– 9.30	—	0.206	43	4.8	—	—
	10.00–11.00	20	2.744	240	11.4	68	Typical x-ray intoxication.
	11.00–12.00	—	2.366	213	11.1	—	—

difference between this type of intoxication and that of acute intestinal obstruction in which a transient impairment of kidney function is constantly found.

TABLE IX.

Large Doses of X-Rays Directly over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 19–117. Adult female setter.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
July 28	38.8	32	67	Normal.
July 28	456 milliampere minutes, x-rays directly over kidneys, other parts shielded; 90 kilovolts; 8 milliamperes.			
July 29	38.8	30	68	Normal.
" 30	38.4	29	66	—
" 31	38.0	27	66	Bread and milk diet.
Aug. 1	37.6	28	63	Dog a little sick.
" 2	37.4	24	63	—
" 3	37.8	—	—	—
" 4	37.4	26	48	—
" 5	37.0	33	32	—
" 6	36.4	—	39	—
" 10	—	33	49	Normal.
" 11	36.5	33	56	—
Aug. 12	512 milliampere minutes, x-rays over both kidneys with other parts shielded; 90 kilovolts; 8 milliamperes.			
Aug. 13	36.3	26	59	—
" 14	36.2	30	—	Dog fed; good condition.
" 15	36.0	30	55	—
" 16	36.1	—	56	—
" 17	36.2	32	48	—
" 18	36.0	38	45	Small amount of vomitus.
" 19	36.0	—	42	Moderate diarrhea.
" 20	35.2	32	58	Clinical improvement.
" 21	35.0	—	—	Normal.

Dog 19–127 (Tables V and VI). Autopsy Record.—Dog died in night of 4th day after exposure to a lethal dose of x-rays given over abdomen with shielding of the kidneys. Autopsy next morning. Kidneys and bladder normal in gross. Histological sections show some postmortem changes in the epithelium of the

convoluted tubules. The stroma and glomeruli are normal. There are no scars. The vessels are filled with blood.

The third type of experiment is represented by the series of observations on Dogs 19–117, 19–129, and 18–115 (Tables IX to XIV).

TABLE X.

Large Dose of X-Rays Directly over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 19–117. Adult female setter.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
July 14	12.50–1.50	—	0.170	30	5.6	—	—
	2.00–3.00	20	1.799	158	11.4	74	Normal.
	3.00–4.00	—	1.323	142	9.3	—	—
July 15	456 milliampere minutes, x-rays over thorax and neck; 90 kilovolts; 8 milliamperes.						
July 18	8.45– 9.45	—	0.160	27	5.9	—	—
	10.00–11.00	20	1.826	159	11.5	67	Normal.
	11.00–12.00	—	1.357	144	9.4	—	—
July 28	456 milliampere minutes, x-rays over kidneys, other parts shielded; 90 kilovolts; 8 milliamperes.						
Aug. 11	2.50–3.50	—	0.160	33	4.8	—	—
	4.00–5.00	20	1.505	173	8.7	56	Not clinically sick.
	5.00–6.00	—	1.182	163	7.3	—	—
Aug. 12	512 milliampere minutes, x-rays over kidneys, other parts shielded; 90 kilovolts; 8 milliamperes.						
Aug. 18	1.45–2.45	—	0.174	38	4.5	—	—
	3.00–4.00	20	1.342	179	7.4	45	Dull. Small amount vomitus.
	4.00–5.00	—	1.316	167	7.8	—	—

In these the kidneys were exposed to huge doses of the x-rays directly while the remainder of the abdomen and the thorax were completely shielded. Large doses were repeatedly given in the case of the first animal while large single doses only were given in the other cases.

TABLE XI.

Large Dose of X-Rays over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 19–129. Adult male mongrel.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
Aug. 6	67.9	29	66	Normal.
" 7	67.4	28	67	—
Aug. 8	512 milliampere minutes, x-rays over thorax; 90 kilovolts; 8 milliamperes.			
Aug. 10	66.7	26	66	Normal.
" 12	65.0	29	63	—
" 14	64.8	25	64	Mixed diet.
Aug. 19	512 milliampere minutes, x-rays directly over kidneys, with other parts shielded; 90 kilovolts; 8 milliamperes.			
Aug. 19	63.0	29	67	Normal.
" 22	61.7	38	53	Slightly dull.
" 24	59.7	46	44	Little vomitus.
" 26	59.3	44	54	Slightly dull.
" 28	59.0	32	58	Normal.

TABLE XII.

Large Dose of X-Rays over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 19–129. Adult male mongrel.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
Aug. 19	8.40– 9.40	—	0.142	29	4.9	—	—
	10.00–11.00	20	1.368	143	9.5	66	Control.
	11.00–12.00	—	1.083	129	8.4	—	—
Aug. 19	512 milliampere minutes, x-rays over kidneys with abdomen shielded; 90 kilovolts; 8 milliamperes.						
Aug. 26	—	—	—	47	—	—	—
	1.00–2.00	20	1.124	161	6.9	47	Slightly dull.
	2.00–3.00	—	0.933	150	6.2	—	—

Dog 19–129 showed no signs of intoxication, whereas No. 19–117 showed a slight reaction and No. 18–115 was markedly intoxicated. It is probable that in the case of No. 18–115 a small portion of the intestine was included in the area of exposure. It is to be noted that the doses given over the kidneys in this group of experiments were at least 25 per cent greater than the lethal dose over the abdomen.

TABLE XIII.

Large Dose of X-Rays over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 18–115. Adult female bulldog.

Date.	Weight.	Blood urea per 100 cc.	Phthalein elimination in 2 hrs.	Remarks.
	lbs.	*mg.*	*per cent*	
Aug. 23	21.5	30	68	Normal.
" 24	20.2	27	66	"
Aug. 27	512 milliampere minutes, x-rays over kidneys with remainder of body shielded; 90 kilovolts; 8 milliamperes.			
Aug. 28	19.4	32	62	Normal.
" 29	19.2	31	54	—
" 30	19.0	33	58	Normal.
" 31	—	—	58	Slightly dull.
Sept. 1	18.3	52	40	Quiet; vomitus and tarry stools.
" 2	17.9	43	—	Tarry stools and vomitus.
Sept. 3	300 milliampere minutes, x-rays over abdomen alone; 90 kilovolts; 8 milliamperes.			
Sept. 4	17.2	36	53	Vomits food; tarry stools.
" 5	16.7	44	46	Weak and dull.
" 8	—	—	—	Found dead in cage.

Dog 19–117 (Tables IX and X). Autopsy Record.—Dog sacrificed under ether Aug. 24 and autopsy done at once. Kidneys and bladder normal in gross. Histological sections show normal glomeruli, vessels, and stroma. There are no scars. Convoluted tubules show normal epithelium. There are a few pale hyaline casts in the collecting tubules of the pyramids.

Dog 19–129 (Tables XI and XII). Autopsy Record.—Dog sacrificed with ether anesthesia Aug. 29 and autopsy done at once. Kidneys show definite chronic nephritis with a large cyst in the left kidney. There is a chronic granular cystitis. Histological sections show a marked cellular pyelitis in both kidneys. There are a few cellular scars in the cortex and some large fibrous scars (arteriosclerotic) involving a considerable area of the cortex and showing tubular and glomerular destruction. There is no evidence of acute nephritis. Outside the scar areas, the glomeruli and tubules appear to be quite normal. This picture is often found in the kidneys of old dogs and cannot in any way be related to the x-ray exposures. This abnormal kidney seems to be no more susceptible to radiation than the normal kidney.

Dog 18–115 (Tables XIII and XIV). Autopsy Record.—Dog found dead in cage Sept. 8 and autopsy done at once. Kidneys are normal in gross. Histological sections show normal glomeruli and vessels. There are a few cellular scars in cortex. The convoluted tubules show a slight postmortem change, but otherwise the tubules are normal.

TABLE XIV.

Large Dose of X-Rays over Kidneys with Abdomen Shielded. Kidney Function Slightly Impaired.

Dog 18–115. Adult female bulldog.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
Aug. 24	2.30–3.30	—	0.153	27	5.6	—	—
	4.00–5.00	20	2.562	197	13.1	66	Control.
	5.00–6.00	—	1.998	181	11.0	—	—
Aug. 27	512 milliampere minutes, x-rays over kidneys with remainder of body shielded; 90 kilovolts; 8 milliamperes.						
Sept. 1	1.30–2.30	—	0.203	52	3.9	—	—
	3.00–4.00	20	2.014	217	9.2	40	Dog is weak. Tarry stools and vomitus.
	4.00–5.00	—	1.683	179	9.4	—	—

Tables IX to XIV inclusive, in which the data of these experiments are recorded, show that the direct exposure of the kidneys to massive doses of the x-rays gives rise to a moderate but definite depression of the kidney function. In Tables XV and XVI are shown the results of two experiments differing slightly from the foregoing in that the kidneys and the intestines were together exposed to large doses of x-rays, the thorax only being protected. A definite decrease in the 2 hour output of phenolsulfonephthalein and a corresponding drop in the urea ratio indicate a moderate impairment of renal function.

TABLE XV.

Lethal Dose of X-Rays over Kidneys and Abdomen.
Renal Function Moderately Impaired.

Dog 19–78. Adult female Airedale.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
Dec. 7	1st	—	—	24	—	—	—
	2nd	20	1.826	152	12.0	69	Control.
	3rd	—	1.594	131	12.1	—	—
Mar. 18	350 milliampere minutes, x-rays over abdomen and kidneys; 95 kilovolts; 8 milliamperes.						
Mar. 20	—	—	—	32	—	55	Slightly dull.
" 21	—	—	—	45	—	40	Intoxication progressing.
Mar. 22	1st	—	—	69	—	—	—
	2nd	20	1.861	258	7.2	38	Marked intoxication; bloody diarrhea.
	3rd	—	1.443	239	6.0	—	—

Dog killed under ether at end of observation.

TABLE XVI.

Lethal Dose of X-Rays over Kidneys and Abdomen.
Renal Function Slightly Impaired.

Dog 19–50. Young adult female.

Date.	Hr.	Urea injected.	Urea excreted per hr.	Blood urea per 100 cc.	Ratio.	Phthalein elimination in 2 hrs.	Remarks.
		gm.	*gm.*	*mg.*		*per cent*	
Feb. 10	1st	—	0.240	29	4.8	—	—
	2nd	20	1.983	180	11.0	66	Control.
	3rd	—	1.529	156	9.8	—	—
Feb. 11	340 milliampere minutes, over abdomen and kidneys; 95 kilovolts; 8 milliamperes.						
Feb. 13	—	—	—	42	—	56	Dull; vomitus and diarrhea.
Feb. 14	1st	—	0.328	78	4.2	—	—
	2nd	20	2.146	280	7.66	40	Frequent diarrhea and vomiting.
	3rd	—	1.543	249	6.19	—	—

From the results of the experiments outlined above we assume that the direct action of the x-rays upon the kidneys is responsible for this depression of activity.

Dog 19–78 (*Tables III and XV*). *Autopsy Record.*—Dog killed under ether at end of experiment given in Table XV. Autopsy done at once but only the renal findings given here. Kidneys are normal in gross. Histological sections show normal glomeruli, vessels, and stroma. There are no scars. Convoluted tubules are normal. The epithelium of the loops of Henle shows a frothy looking protoplasm but this picture is seen in control dogs.

Dog 19–50 (*Tables IV and XVI*). *Autopsy Record.*—Dog killed under ether anesthesia at end of last experiment (Table XVI) and autopsy at once. Only the renal findings are given here. Kidneys and bladder normal in gross. Histological sections show normal glomeruli and vessels. There are a few small cellular scars in the cortex. Tubules are normal.

Reference to the autopsy findings in the various dogs used in the experiments shows that no constant anatomical change is seen in the kidneys which would serve to distinguish them from a series of normal control kidneys chosen at random. We did not use the refined histological technique recommended by Warthin, but we are inclined to believe that much of the histological evidence submitted in favor of x-ray injury of the kidney is due to kidney abnormalities present *before the x-ray exposures.* Such abnormalities would be revealed by a careful study of suitable controls.

SUMMARY.

Our experiments give no support to the current belief that an x-ray nephritis may be produced by direct or indirect action of the hard Roentgen rays.

Moderate doses of x-rays given *repeatedly* over considerable periods of time have no demonstrable influence on renal function or renal structure.

With x-ray exposures of the abdomen and shielding of the kidneys fatal intoxication may be produced without the slightest disturbance of kidney function as measured by the ability of the kidney to eliminate phenolphthalein and urea.

Large doses of the x-rays given directly over the kidney may cause a slight but distinct lowering of renal function which lasts for a period of a few days. We have been unable to recognize any corresponding histological change.

We feel that the usual therapeutic doses of the x-rays can be given over the kidneys without apprehension. The renal tissue is much more resistant to x-rays than is the epithelium of the small intestine.

BIBLIOGRAPHY.

1. Addis, T., and Watanabe, C. K., *J. Biol. Chem.*, 1916–17, xxviii, 251.
2. Buschke, A., and Schmidt, H. E., *Deutsch. med. Woch.*, 1905, xxxi, 495.
3. Edsall, D. L., and Pemberton, R., *Am. J. Med. Sc.*, 1907, cxxxiii, 286.
4. Hall, C. C., and Whipple, G. H., *Am. J. Med. Sc.*, 1919, clvii, 453.
5. Heymann, P., *Fortschr. Geb. Röntgenstrahlen*, 1908, xii, 212.
6. von Jaksch, R., *Z. klin. Med.*, 1907, lxiv, 316.
7. Krause, P., *Münch. med. Woch.*, 1906, liii, 1745.
8. Krause, P., and Ziegler, K., *Fortschr. Geb. Röntgenstrahlen*, 1906, x, 126.
9. Linser, P., and Helber, E., *Deutsch. Arch. klin. Med.*, 1905, lxxxiii, 479.
10. McQuarrie, I., and Whipple, G. H., *J. Exp. Med.*, 1919, xxix, 397.
11. McQuarrie, I., and Whipple, G. H., *J. Exp. Med.*, 1919, xxix, 421.
12. Přibram, H., and Rotky, H., *Z. exp. Path. u. Therap.*, 1909, vi, 75.
13. Rosenstern, I., *Münch. med. Woch.*, 1906, liii, 1063.
14. Rowntree, L. G., and Geraghty, J. T., *Arch. Int. Med.*, 1912, ix, 284.
15. Schleip, K., and Hildebrand, W., *Münch. med. Woch.*, 1905, lii, 396.
16. Stephan, R., *Strahlentherapie*, 1920, xi, 517.
17. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 141.
18. Warthin, A. S., *Am. J. Med. Sc.*, 1907, cxxxiii, 736.

THE RELATION OF THE HYPOPHYSIS TO ANTIBODY PRODUCTION.

By ELLIOTT C. CUTLER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, August 30, 1921.)

The important part that many of the endocrine organs play in development and function suggests the possibility that they may also enter into the mechanism of the body which develops resistance to infection. Up to the present time there is little positive evidence that such is the case. It was felt, however, that certain of the more inaccessible glands of internal secretion, whose function in relation to immunity had not been studied, might yield valuable information, and this study of the pituitary gland was made as part of a general investigation into this field (6).

The experimental work so far has been chiefly of a physiological nature concerning the part the pituitary plays in growth, metamorphosis, sugar tolerance, and sexual activity, and its relation to acromegaly, dyspituitarism, and diabetes mellitus. The functions of the two parts of the gland have been elaborately defined (13, 16). More recently the interrelationship of this gland to the other ductless glands has been much discussed. That total hypophysectomy is incompatible with life[1] would seem to be proved by the work of Cushing and his collaborators (3, 4) and Paulesco (17). The striking physical changes in growth, development, nutrition (3), and general metabolism (2) with partial hypophysectomy (1) and by feeding pituitary gland extract (9, 10, 20) are now accepted. We have not been able to find, however, any direct, quantitative, experimental studies of the relation of the hypophysis to infectious diseases. Aschner (1) thought his partially hypophysectomized dogs succumbed more easily to disease than normal dogs, and certainly many contracted pneumonia. Cushing's experiments (3) seem to bear this out. There are, however, no clinical observations that would seem to indicate a lessened or increased resistance to infectious disease with pituitary disorders.[2] Gay and Rusk (7) in a study of the locus of antibody production draw no definite conclusion, but the evidence would seem to indicate that the spleen and the lymphatic system play the most important part. The work of Murphy and his associates (15), Hektoen (11), Hektoen and Curtis (12), and Morris and Bullock (14) appears to corroborate this view. The experiments of Topley (19) appear to disprove the contention that antibodies are formed at the point of inoculation.

---

[1] The work of Gemelli (8), Aschner (1), and Handelsmann and Horsley (10) disputes this, but their extirpation methods were so injurious and uncontrolled as to appear unreliable. Our own studies on the guinea pig agree with those of Paulesco and Cushing.

Problem and Methods.

Guinea pigs were selected as the experimental animal because of their already standardized reactions in immunological studies. An operative technique was elaborated for partial hypophysectomy. In such animals the serum reactions of typhoid agglutination, hemagglutination, and hemolysis were studied. These studies were completed in the following series.

Series 1. The Production of Antibodies after Hypophysectomy.—Partial hypophysectomized, control operation animals and normal controls were immunized to *Bacillus typhosus*. Subsequent titers indicated the degree of immunity produced.

Series 2. The Effect of Hypophysectomy on Antibody Production.—This was measured by the change in height of the antibody curve. Guinea pigs were immunized to (1) *Bacillus typhosus* and (2) hen red blood corpuscles, and then submitted to partial hypophysectomy. Normal and control operation animals were used. Titration of agglutinins and hemolysins was carried out at intervals before and after operation.

Series 3. The Effect of Feeding and Injecting Pituitary Extract on the Production of Antibodies.—Animals were immunized, then fed continuously pituitary extract or submitted to repeated injection of the extract, and their subsequent titers determined.

Operative Technique.

Adult guinea pigs weighing from 300 to 400 gm. were used. In such animals the pituitary is relatively large, weighing about 0.025 gm. (Vanderburgh (21)). It lies in a shallow dural pocket and has the characteristic histologic appearance of other mammalian hypophyses. After some difficulties the following operative attack was chosen as giving an approach through which a definite portion of the gland could be removed and in which success was obtained in about 75 per cent of the animals used. The animal was placed on an electrically heated pad, kept at a fixed temperature, and fastened down to a small raised table. Anesthesia was induced by means of a fine rubber tube passed into the posterior pharynx through which a small pump forced ether vapor (5). Morphia 0.002 gm. and atropine 0.004 gm. were given 15 to 30 minutes before anesthesia. After shaving the head and cleaning the scalp with 1:1,000 bichloride a wet gauze covering was placed over the head and a sterile towel over the body of the animal. The gauze over the vertex of the skull was cut and an incision in the skin was made from just behind the eyes to the occipital protuberance and carried through to the bone. The tissue flaps on both sides were then dissected up and held away by the weight of a single hemostat. The parietal bone was perforated and all the bone over both hemispheres was removed as far anteriorly as the fronto-parietal synostoses and posteriorly to the occipital bone. On the right side the decompression was carried to the temporoparietal juncture and on the left well into the temporal bone. The dura was left intact and the longitudinal sinus which lies in it, free from the bone, remained uninjured. The dura was then incised low down in the left temporal region, a small spatula introduced beneath the temporal lobe, and the brain elevated and slightly dislocated towards the right side. (The removal of bone on the right side was done to permit this.) A view was thus obtained of the pituitary fossa, the small pinkish body lying surrounded on both sides and posteriorly by large venous sinuses. The stalk enters at the anterior end of the gland, running slantwise to the posterior region. The dura over the middle of the hypophysis was then nicked with a small hook knife and with a small double spoon forceps fragments of the gland were removed. Unless the sinus was injured, which was fatal, there was little hemorrhage. Warm normal saline solution on cotton pledgets was used for sponging and hemostasis. After removal of the gland fragment, the brain was allowed to return to its position and the skin carefully sutured with two layers of fine interrupted silk sutures. This was covered by a cocoon dressing. The operation consumed between 30 and 45 minutes.[3] Control animals in which the same operation, without removal of the pituitary, was performed, were prepared. The above procedure was carried out through the step in which the dura over the sella was cut and then closure completed, thus subjecting the control animals to approximately the same amount of trauma and anesthesia.

---

[2] Personal communication from Dr. Harvey Cushing.

Total hypophysectomy proved fatal, the animals almost at once entering into a lethargic, partly comatose state, refusing food and water and dying in 24 to 48 hours. All fragments removed were studied histologically, and no animals are reported in which there was not this proof that at least one-half of the gland, which always consisted of fragments of both pars anterior and posterior, was removed. Autopsy controls were used to establish further the efficacy of this method of attack. The surviving animals within 24 hours made excellent recoveries, but lost 50 to 100 gm. in weight in the first few days. After this they regained weight and appeared, except for a rare ocular palsy, as active and vigorous as the normal controls. In many cases the gain in weight was rapid and even above that of the normal animals.

---

[3] The chief complication in recovered animals was an ocular palsy, the result apparently of injuring the third cranial nerve which lies practically on the hypophysis but which we attempted to push to one side before the final step of enucleation.

Technique of the Serum Reactions.

Typhoid Agglutinins.—The Sen strain of *B. typhosus* was used.[4] It had been grown on artificial media for years but still combined a ready agglutinability with a high toxicity for guinea pigs. It was carried along throughout these experiments by repeated daily transplants on agar and bouillon with occasional plating to determine its purity. A vaccine was prepared by suspending the 24 hour growth in six Blake bottles in salt solution, killing the organisms by adding chloroform, drying them in a vacuum, and then grinding up the bacteria in a sterile mortar. Weighed quantities were then suspended in normal salt solution and tricresol was added for maintaining sterility. Experimentation with vaccine and living organisms determined that the greatest reaction was produced by the following inoculations given at 3 and 4 day intervals: first injection, 0.002 gm. of vaccine subcutaneously; second injection, 0.002 gm. intraperitoneally; third injection, $\frac{1}{30}$ of a 20 hour growth of living organisms on an agar slant intraperitoneally; and fourth injection, $\frac{1}{30}$ of a 20 hour growth of living organisms on an agar slant intraperitoneally. By the 10th day after the last injection agglutinin titers ranged from 1:1,000 to 1:6,000. In Text-figs. 1 and 2 the normal control curve represents the average of over twenty-five animals. In performing agglutination tests 1 drop of a salt suspension of a 24 hour growth of the bacteria on agar was added to 1 cc. of successive dilutions of serum. Serum was obtained by cardiac puncture, 4 cc. of blood being withdrawn, and the serum inactivated. Titration was recorded after 2 hours at 37°C., after 2 hours at room temperature, and after the tubes had been in the ice box over night. 4 cc. of blood proved sufficient for both agglutination and complement fixation tests. The latter tests, however, although completed with almost all animals used, are not reported since the concentration of complement fixation bodies was not sufficient to ensure reliable comparative results.

---

[4] This strain was obtained from Dr. Carroll G. Bull.

Hemagglutinins and Hemolysins.—Another series of guinea pigs with normal controls, partially hypophysectomized controls, and operation controls was immunized to hen red blood corpuscles for the study of hemagglutinins and hemolysins. At 4 day intervals all animals were given intraperitoneally 1 cc. of washed hen corpuscles made up to the original blood volume. At the end of 1 week serum was obtained and the quantity of reaction determined. In testing for hemagglutinins 1 drop of a 10 per cent suspension of washed hen corpuscles was added to 1 cc. volumes of successively diluted, inactivated serum. Readings were made as with the typhoid studies. For hemolysins the usual technique of the Wassermann reaction was followed with 0.25 cc. volumes of inactivated serum, fresh guinea pig complement 1:10, a 5 per cent suspension of hen corpuscles, and 0.5 cc. of saline solution. The tubes were then incubated for 1½ hours, being shaken after 30 minutes and 1 hour. Readings were made after 1½ hours and after standing in the ice box over night. The usual controls were performed with each test.

The several series were performed with the above methods and complement deviation studies were also made. The latter, however, are not reported because of the low and variable nature of the titers resulting; it appears probable that for this test insufficient antibodies are manufactured. Operative difficulties now and then caused the loss of valuable animals but a sufficient number was always used in each experimental unit to ensure a satisfactory number of complete studies. As a rule, the experimental unit consisted of two normal animals, two partially hypophysectomized animals, and two operative control animals for Series 1. Series 2 experiments, however, were usually started with eight guinea pigs, since the operation was completed only when an immunity had been established and titers determined, and unless sufficient animals were provided the loss of a guinea pig at operation would vitiate that particular experimental unit. Before commencing with typical experimental units, the operative technique was perfected and a supply of hypophysectomized and control operation animals provided. The agglutinin, hemolysin, and hemagglutinin reactions were studied in normal guinea pigs and the quantitative reaction was well standardized. The immunity production in partially hypophysectomized and operative control animals showed no appreciable difference in these preliminary studies. In the actual experimental units guinea pigs as near of a size and kind as possible were chosen. Because of the reported interrelation of the pituitary body with the sex glands (9) and the reported increased reaction in menstruating women to typhoid vaccine, preliminary studies were made which ruled out any appreciable influence that such sexual periods might have on antibody production. Neither menstruation[5] nor the pregnant state appeared to have any influence on the antibody curve.

EXPERIMENTAL.

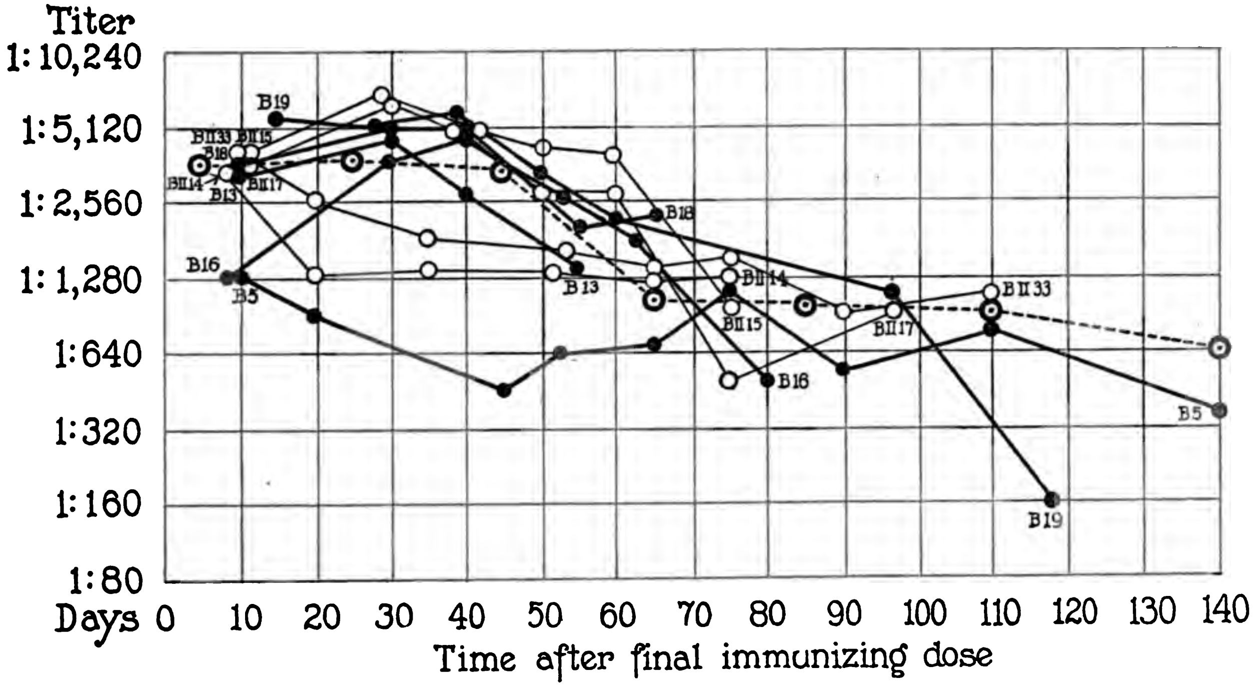
Series 1. The Production of Agglutinins after Hypophysectomy.—Guinea pigs, in which partial hypophysectomy[6] and a control operation[6] had been performed were immunized to *Bacillus typhosus* by the above method. Their agglutinin titers were recorded from the 7th to the 10th day after the last injection and followed every week or 2 weeks. Unoperated control animals were carried along in each experimental unit, but their course ran such a comparatively parallel curve to the large number of normal animals studied previously that an average line has been used for Text-figs. 1 and 2 in order to avoid confusion. Text-fig. 1 represents a group of hypophysectomized animals with operated controls compared to the normal average agglutinin curve. The general curve in the hypophysectomized and control operation animals shows a parallelism to the normal controls and needs no comment. The repetition of such experiments brought the same results. Partial hypophysectomy apparently has no influence on the subsequent production of typhoid agglutinins in guinea pigs.

Series 2. The Effect of Hypophysectomy on Antibody Production.—Three sets of experiments were performed. In Class 1 the agglutinin titer in animals immunized to *Bacillus typhosus* was studied before and after hypophysectomy, in Class 2 the hemagglutinin titer, and in Class 3 the hemolysin production. Normal and operated controls were used. For each experimental unit eight animals were used. All were immunized, two were saved for normal controls and three each submitted to partial hypophysectomy or the control operation.

---

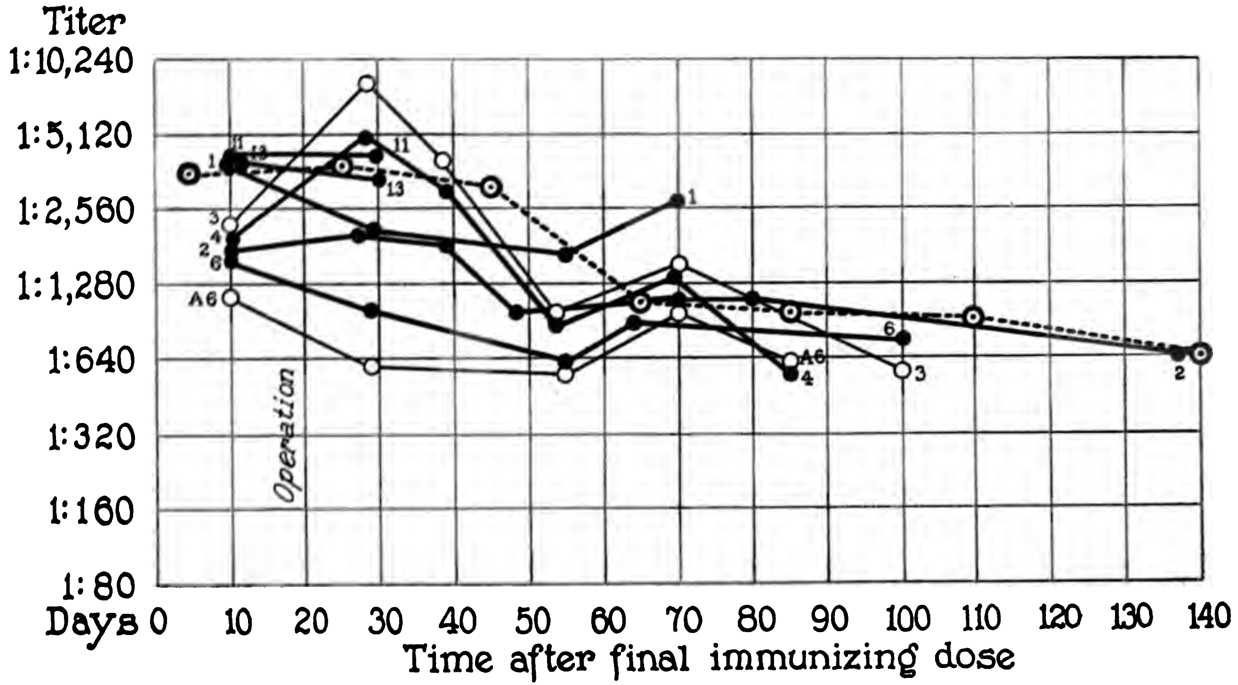
[5] The guinea pig has a definite menstrual cycle (18).

[6] At least 8 days after the operation, when the animals were entirely recovered.



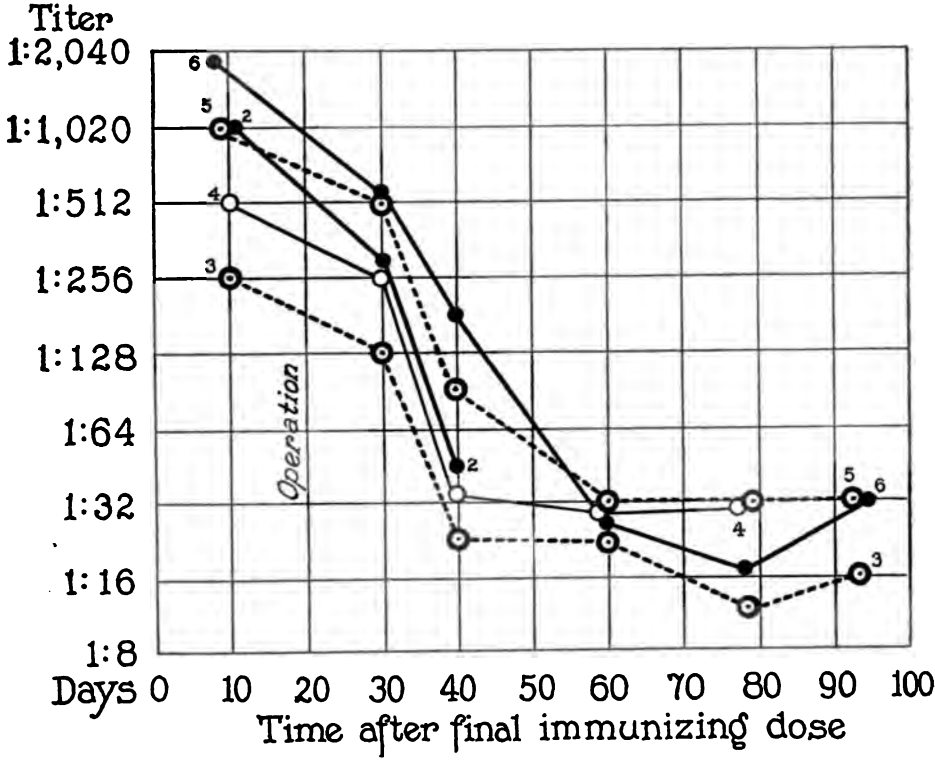
In Text-figs. 1 to 4 the following curves are used. ━━━━ Partially hypophysectomized animals.
———— Control operation animals. ------------ Normal control animals.

TEXT-FIG. 1. Series 1. Typhoid agglutinin titers of animals immunized after hypophysectomy.



TEXT-FIG. 2. Series 2. Typhoid agglutinin titers before and after hypophysectomy.

Text-fig. 2 represents the study of such an experiment in Class 1, in which the animals were immunized to *Bacillus typhosus*. No special comment is necessary since all titrations fall practically within normal limits.

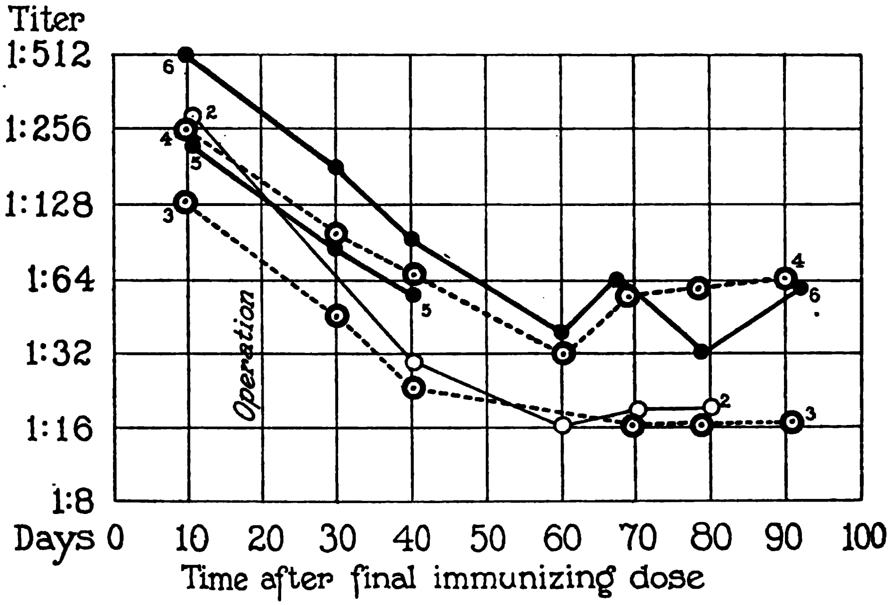


TEXT-FIG. 3. Series 2. Hemagglutinin titers before and after hypophysectomy.

Text-figs. 3 and 4 represent the studies in Classes 2 and 3. Here, too, the parallel of operated animals to normal animals is striking. Repeated experimental units showed no appreciable variation.

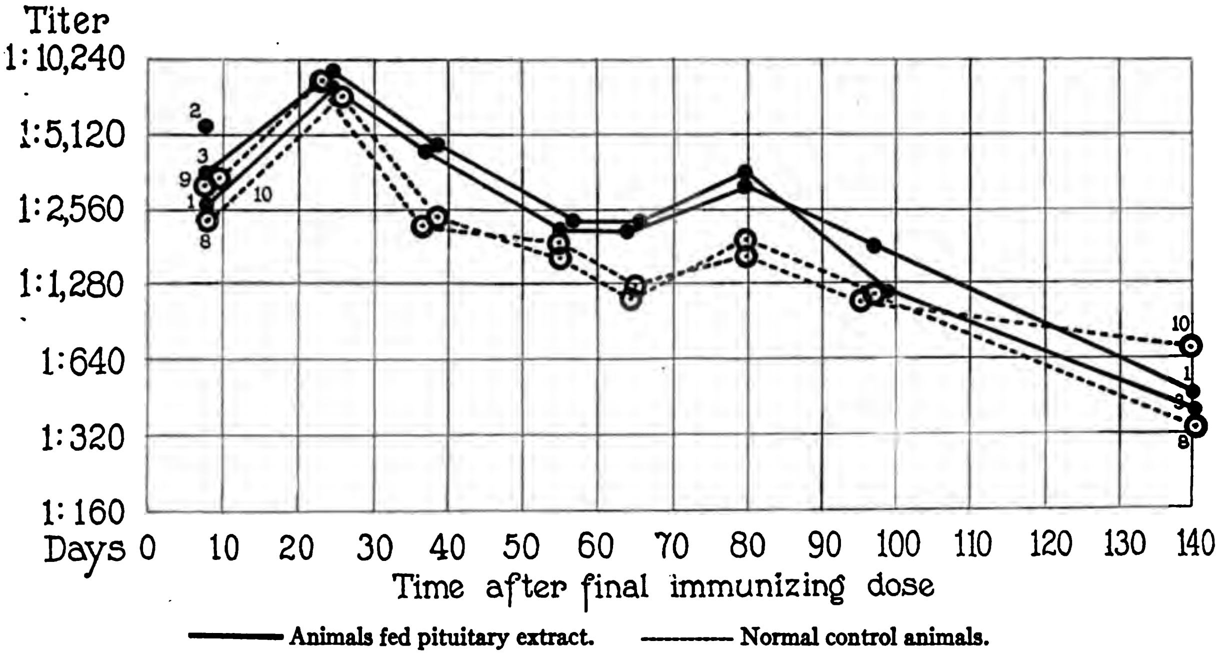
These experiments would seem to indicate that partial hypophysectomy has no influence on the maintenance of specific agglutinins for *Bacillus typhosus*, hemagglutinins, or hemolysins in the guinea pig.

Series 3. The Effect of Feeding and Injecting Pituitary Extract on Antibody Production.—In the feeding experiments six animals were selected and immunization was carried out as described above. Beginning on the day of the first inoculation all animals were fed two medicine droppers full of milk. To the milk of three animals 0.13 gm. of the Burroughs Wellcome whole pituitary gland extract was added. The feeding of milk and of pituitary extract in the same amount of milk was carried out daily until the experiment was finished. This was the method of feeding gland extract adopted by Goetsch (9) and proved very satisfactory, the animals taking their ration with evident relish. The control animals were fed milk to obviate any criticism of extra nourishment vitiating or influencing the experiments. Beginning 9 days after the last inoculation the agglutinin titer was studied, such studies being then repeated at weekly or biweekly intervals. Text-fig. 5 represents an experimental unit of the above description. The parallelism of normal and pituitary-fed animals is striking.



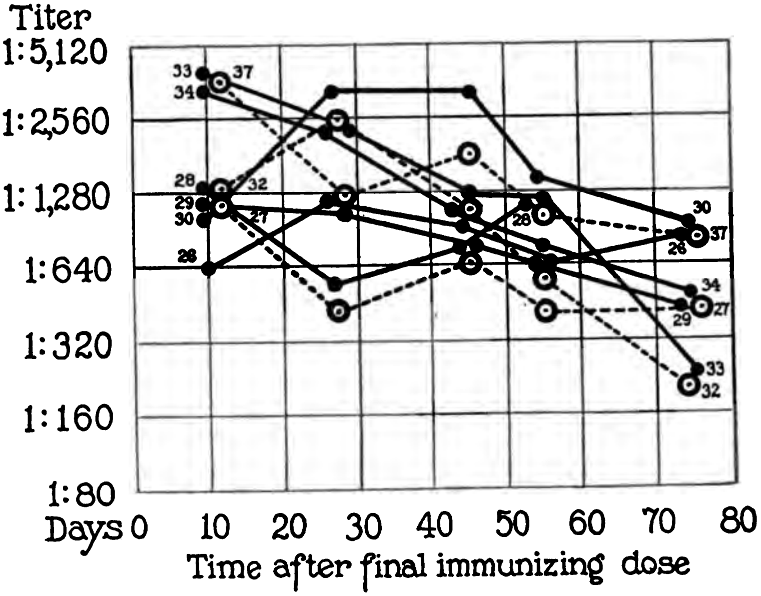
TEXT-FIG. 4. Series 2. Hemolysin titers before and after hypophysectomy.

In another group of animals the gland was given by intraperitoneal injection. 1 gr. of Burroughs Wellcome whole gland extract



TEXT-FIG 5. Series 3. The effect of feeding pituitary extract on the typhoid agglutinin titer. In two animals, only one determination was made because they died from cardiac puncture the day of the first titration.

tablets was used dissolved in 1 cc. of normal saline solution. 1 cc. of normal saline solution was injected at corresponding intervals into the controls. Nine animals were used in these tests, three for controls and six for injection. The animals were first immunized to *Bacillus typhosus*. On the 10th day following the last inoculation their agglutinin titer was determined. This was repeated in 18 days. 10 days later the injection of pituitary extract and salt solution was



——— Animals injected with pituitary extract.
- - - - - Normal control animals.

TEXT-FIG. 6. Series 3. The effect of repeated intraperitoneal injections of pituitary extract on the typhoid agglutinin titer.

begun and repeated every other day for 3 doses, following which the agglutinin titer was again done. Later the pituitary extract injections were repeated and again subsequent agglutinin titrations carried out. Such an experiment is reported in Text-fig. 6. Further studies varying the amount of extract injected and the intervals between injections were carried out, but no appreciable changes were noticed. As seen in Text-fig. 6 the animals injected with pituitary ran a titer approximate to that of the controls.

SUMMARY.

An operative technique was evolved permitting successful partial hypophysectomy in guinea pigs.

Such animals, when immunized to *Bacillus typhosus*, produced specific agglutinins in the same quantity and at the same rate as unoperated and operation controls immunized at the same time and by the same method.

In guinea pigs previously immunized to *Bacillus typhosus* and hen red blood corpuscles partial hypophysectomy had no effect on the continued production and persistence of typhoid agglutinins, hemagglutinins, and hemolysins.

In guinea pigs immunized to *Bacillus typhosus* both the continued ingestion and the intraperitoneal injection of the whole pituitary gland extract (Burroughs Wellcome) had no effect on the subsepuent agglutinin titers as compared to that of normal animals.

The experiments would appear to show either that the hypophysis does not play an important direct or indirect part in the production of and persistence in the blood of typhoid agglutinins, hemagglutinins, and hemolysins, or that the amount of hypophysis left behind in the operation in order to maintain life is adequate also to exercise the degree of functional influence on these processes which the entire hypophysis conceivably exercises.

BIBLIOGRAPHY.

1. Aschner, B., Demonstration von Hunden nach Exstirpation der Hypophyse (kurze Mitteilung), *Wien. klin. Woch.*, 1909, xxii, 1730; 1910, xxiii, 572.
2. Benedict, F. G., and Homans, J., The metabolism of the hypophysectomized dog, *J. Med. Research*, 1912, xxv, 409.
3. Crowe, S. J., Cushing, H., and Homans, J., Experimental hypophysectomy, *Bull. Johns Hopkins Hosp.*, 1910, xxi, 127.
4. Cushing, H. W., The pituitary body, Philadelphia and London, 1912.
5. Gates, F. L., A valve to regulate the delivery of air and ether vapor in any proportion, *J. Exp. Med.*, 1917, xxvi, 41.
6. Gates, F. L., Antibody production after partial adrenalectomy in guinea pigs, *J. Exp. Med.*, 1918, xxvii, 725.
7. Gay, F. P., and Rusk, G. Y., Studies on the locus of antibody formation, *Tr. XVth Internat. Cong. Hyg. and Democ.*, 1912, ii, 328.
8. Gemelli, A., Ulteriore contributo alla fisiologia dell ipofisi, *Folia neuro-biol.*, 1908–09, ii, 167.

9. Goetsch, E., The influence of pituitary feeding upon growth and sexual development, an experimental study, *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 29.

10. Handelsmann and Horsley, V., Preliminary note on experimental investigations on the pituitary body, *Brit. Med. J.*, 1911, ii, 1150.

11. Hektoen, L., The influence of the x-ray on the production of antibodies, *J. Infect. Dis.*, 1915, xvii, 415.

12. Hektoen, L., and Curtis, A. R., The effect on antibody production of the removal of various organs, *J. Infect. Dis.*, 1915, xvii, 409.

13. Howell, W. H., The physiological effects of extracts of the hypophysis cerebri and infundibular body, *J. Exp. Med.*, 1898, iii, 245.

14. Morris, D. H., and Bullock, F. D., The importance of the spleen in resistance to infection, *Ann. Surg.*, 1919, lxx, 513.

15. Murphy, Jas. B., Factors of resistance to heteroplastic tissue-grafting. Studies in specificity. III, *J. Exp. Med.*, 1914, xix, 513. Murphy, Jas. B., and Ellis, A. W. M., Experiments on the rôle of lymphoid tissue in the resistance to experimental tuberculosis in mice, *J. Exp. Med.*, 1914, xx, 397. Murphy, Jas. B., and Morton, J. J., The lymphocyte in natural and induced resistance to transplanted cancer. II. Studies in lymphoid activity, *J. Exp. Med.*, 1915, xxii, 204. Murphy, Jas. B., Nakahara, W., and Sturm, E., Relation between time and extent of lymphoid stimulation induced by physical agents and degree of resistance to cancer in mice, *J. Exp. Med.*, 1921, xxxiii, 423.

16. Oliver, G., and Schäfer, E. A., On the physiological action of extracts of pituitary body and certain other glandular organs, *J. Physiol.*, 1895, xviii, 277.

17. Paulesco, N. C., L'hypophyse du cerveau. I. Physiologie, Paris, 1908.

18. Stockard, C. R., and Papanicolaou, G. N., A rhythmical "heat period" in the guinea pig, *Science*, 1917, xlvi, 42.

19. Topley, W. W. C., Some experiments bearing on the local formation of antibodies, *Proc. Roy. Soc. Med.*, 1920, xiii, pt. 3, path. sect., 144.

20. Uhlenhuth, E., Experimental production of gigantism by feeding the anterior lobe of the hypophysis, *J. Gen. Physiol.*, 1920–21, iii, 347.

21. Vanderburgh, C. M., The hypophysis of the guinea pig, *Anat. Rec.*, 1917, xii, 95.

STUDIES UPON EXPERIMENTAL MEASLES.

I. The Effects of the Virus of Measles upon the Guinea Pig.

By CHARLES W. DUVAL, M.D., and RIGNEY D'AUNOY, M.D.

(*From the Laboratories of Pathology of Tulane University, New Orleans.*)

(Received for publication, August 15, 1921.)

Investigations dealing with the nature of the microorganismal excitants of the exanthematous diseases have been numerous during the past two decades, and the present point of view relates the latter to the so called filterable viruses. It is obvious that more precise and definite knowledge of these agents is greatly to be desired.

The nature of the virus of measles has received little attention from the standpoint of research since the transmission experiments of Hektoen[1] showed the existence of the virus in the circulating blood, and those of Goldberger and Anderson[2] proved that the virus is a filter passer. The recent work of Blake and Trask[3] has both extended and confirmed previous knowledge and has appeared at a strategic time when the studies of Sellards[4] had shaken confidence in the earlier experimental findings.

In the investigation of the virus of measles or of the exanthemata as a whole, it is a striking fact that the ape, chimpanzee, and the monkey especially, have been the experimental animals of choice, and they have been chosen because of the greater likelihood of reproducing in them a clinical picture analogous to that of the human disease.

---

[1] Hektoen, L., Experimental measles, *J. Infect. Dis.*, 1905, ii, 238.

[2] Goldberger, J., and Anderson, J. F., The nature of the virus of measles, *J. Am. Med. Assn.*, 1911, lvii, 971.

[3] Blake, F. G., and Trask, J. D., Jr., Studies on measles. I. Susceptibility of monkeys to the virus of measles, *J. Exp. Med.*, 1921, xxxiii, 385.

[4] Sellards, A. W., and Wentworth, J. A., Insusceptibility of monkeys to inoculation with blood from measles patients, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 57. Sellards, A. W., Insusceptibility of man to inoculation with blood from measles patients, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 257; The reaction of monkeys to the inoculation of measles blood, 311.

Since the work of Inada and Ido[5] on hemorrhagic jaundice, and that of Noguchi on yellow fever, it is evident that the lower type of laboratory animal, namely, the guinea pig, is to be regarded an important host for even highly specialized parasitic microorganisms. The observations of Noguchi[6] on the effects of the virus of yellow fever in the guinea pig have shown that a detailed study of the temperature, the leucocytes, and the clinical phenomena, comprises in this animal an important basis for establishing the existence of infection. This class of observations may be regarded as having contributed to the discovery of *Leptospira icteroides*. While there is no intention of intimating that a leptospira may be the incitant of measles, it appeared to us that if such phenomena could be induced in the guinea pig by the inoculation of measles virus they would provide a far more practical method of handling the experimental measles problem. The use of guinea pigs in experiments on measles has heretofore been limited, and the results have proved unsatisfactory for the reason that the obvious clinical effects looked for were not detected.

As Hektoen and later other investigators demonstrated the presence of the active agent of measles in the circulating blood during the height of the disease, we chose this medium as the virus material with which to attempt transmission to and propagation in the guinea pig. An abundance of material was available, as, during the months of December, 1920, and January and February, 1921, an epidemic of measles prevailed in New Orleans, providing many suitable cases for observation.

EXPERIMENTAL.

The object of the experiments was the determination of the possibility of transmission of measles virus from man to the guinea pig. If transmission could be successfully accomplished, propagation of the virus by passage from guinea pig to guinea pig and a study of the effect of such passage on virulence, together with a study of the relative infectivity of the passage virus, were next to be attempted. Finally, the plan included the determination of the relative infectivity of blood obtained in various stages of the human disease; namely, prodromal, preeruptive, eruptive, and convalescent.

---

[5] Inada and Ido, A report on the discovery of the causal organism (a new species of spirochæta) of Weil's disease, *Tokyo Ijishinshi*, 1915, No. 1908. Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., The etiology, mode of infection, and specific therapy of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiii, 377.

[6] Noguchi, H., Etiology of yellow fever. II. Transmission experiments on yellow fever, *J. Exp. Med.*, 1919, xxix, 565.

The blood taken for clinical study was collected under aseptic precautions by means of all glass syringes, immediately placed in sterile flasks containing glass beads, and defibrinated by a rotary motion. Within 15 to 30 minutes after the blood was removed and defibrinated a part was injected into the circulation of the guinea pig. The animal inoculations were made by intracardiac injection of 1 to 2 cc. of material. The guinea pigs were anesthetized with ether and cardiopuncture was accomplished with a No. 24 gauge needle. In order to be certain that the inoculated blood was deposited directly into the heart chambers, removal of a small portion of blood was performed by syringe suction before the introduction of the infecting material.

The method of study after inoculation consisted in twice daily taking of rectal temperatures, daily counting of the total leucocytes, and close inspection of the animals for signs and symptoms of illness during a period of 5 weeks observation. Animals sacrificed at the height of the reaction and those dying subsequent to inoculation were autopsied and a gross and microscopic study of the tissues carried out. Cultures were made from heart's blood and internal organs to determine the presence of intercurrent infection.

The leucocytic count of normal guinea pigs has been made the subject of fairly comprehensive study. Thus, Bethe[7] gives the average number as 7,240 per c. mm., Kurloff[8] as 12,600 per c. mm., and more recently Burnett[9] as 10,897. Hussey[10] is in entire disaccord with these investigators in that he gives 5,247 as the average count on an unstated number of animals.

---

[7] Bethe, M., Beiträge zur Kentniss der Zahl- und Massverhältnisse der rothen Blutkörperchen, *Morphol. Arb.*, 1892, i, 207.

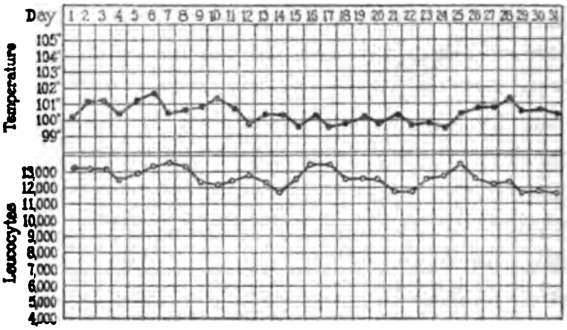
[8] Kurloff, cited by Ehrlich, P., and Lazarus, A., Die Anæmie, in Nothnagel, H., Specielle Pathologie und Therapie, Vienna, 1901, viii, 56.

[9] Burnett, S. H., A study of the blood of normal guinea pigs, *J. Med. Research*, 1904, xi, 537.

[10] Hussey, R. G., General leucocytic response of the guinea pig during the reaction of artificial immunity in experimental tuberculous infection, *J. Exp. Med.*, 1921, xxxiii, 337.

We have not been able to find in the literature any extensive reference to the normal temperature limits of guinea pigs. We accordingly decided to construct curves of the rectal temperatures of these animals, in order to furnish a basis for the determination of pyrexia.

Experiment 1 (*Preliminary*).—Thirty guinea pigs were used in the experiment, temperatures being determined daily for 31 days. For sake of completeness, leucocytic determinations were made at the same time. Text-fig. 1 represents the composite curves constructed as a result of the experiment.

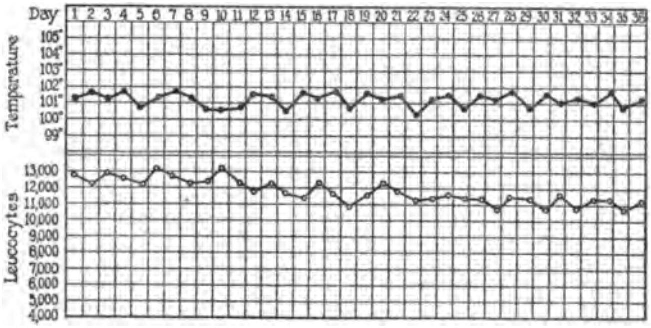


TEXT-FIG. 1. Composite temperature and leucocytic charts of thirty normal guinea pigs.

The results show that the average temperature of the animals in our series, was 100.7°F., the limits being 100° and 101.4°F. In the main experiments, we disregarded as an elevation of temperature any reading below 102°F. The leucocytes for our series averaged 12,250, the limits being 10,750 and 13,750.

Experiment 2 (*Preliminary*).—Since defibrinated blood from cases of human measles was to be injected into the circulation of guinea pigs, it appeared desirable to establish whether or not any reactionary objective signs, temperature increase, or leucocytic changes could be induced by the injection of normal defibrinated blood. Accordingly intracardiac injections were performed on a series of fifteen guinea pigs, defibrinated blood from adults and children being given in quantities varying from 0.5 to 5 cc. Temperature and leucocytic curves constructed in Text-fig. 2 show the effects of these inoculations.

It thus appears that normal defibrinated blood when injected into the circulation of guinea pigs occasions no pyrexial reaction or leucocytic changes. Furthermore no objective signs of illness were to be noted in any of these animals. A number of guinea pigs were sacrificed at periods ranging from 7 to 20 days after inoculation and a histopathological study was made of the various organs. In no case were lesions discernible, except small localized areas of congestion and atelectasis in the lungs, to which we paid no special attention, for as pointed out by Maitland, Cowan, and Detweiler,[11] such changes are agonal and frequently occur following an anesthetic and death in the guinea pig.



TEXT-FIG. 2. Effects of intracardiac inoculation of normal human defibrinated blood upon temperature and leucocytes of guinea pigs.

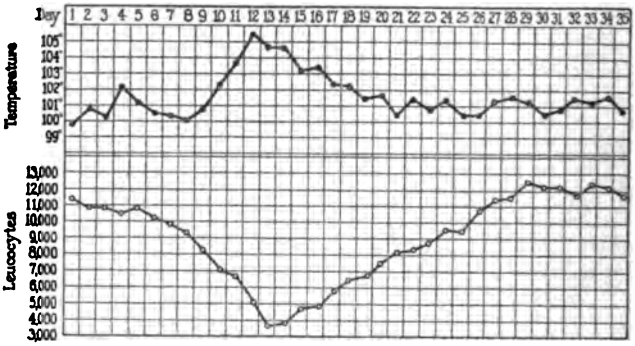
Experiment 3.—The purpose was to determine whether the virus of measles could be directly transmitted from man and propagated in the guinea pig. Blood from seven different cases of measles in the eruptive stage of the disease was employed as the inoculating material. Six of the cases were children ranging in age from 4 to 10 years, and one was an adult. For each experiment of the series, six guinea pigs were used, four for inoculation and two for control. The test animals all received 2 cc. of defibrinated blood containing virus directly into the cardiac circulation, while the controls were given a like quantity of normal blood. In each instance the blood was cultured on ordinary media at the time of injection to exclude bacterial contamination.

---

[11] Maitland, H. B., Cowan, M. L., and Detweiler, H. K., *Brit. J. Exp. Path.*, 1921, ii, 8.

Of the twenty-eight guinea pigs injected with virus, twenty subsequently showed unmistakable reactions, alike in all respects, which could only be interpreted as indicative of transmission of the infecting material. The control animals remained normal throughout the entire period of the experiment. In general, the effect of the blood containing virus upon the guinea pigs was characterized by a gradual rise of from 2–4° in the temperature, beginning on the 11th to 14th days after inoculation, the animals in the interim remaining well to all outward appearances.

The average number of guinea pigs in each of the seven series that gave a definite reaction was three, or 75 per cent. Perhaps a greater percentage of the animals of this series reacted, but we considered as reactions only those which showed a rise in temperature above 102°C. Coincident with the temperature elevation the leucocytes dropped until a decided leucopenia, as low as 4,000 cells per c.mm. in a number of instances resulted. The fall in the circulating leucocytes was a more constant sign of the reaction than the temperature rise. While in the majority of the reacting animals the temperature rise occurred coincidently with the leucocytic drop, in six animals there occurred a well marked leucopenia by the 12th day with no coincident temperature rise. It is noteworthy that in the majority of the animals reacting there occurred an initial rise in the temperature on the 2nd or 3rd day after inoculation, unaccompanied by any fall in the leucocytes. This pyrexia was maintained only for a period of 24 hours and usually represented an elevation of 1°F. At first we attributed this initial rise to the foreign protein inoculated; however, animals receiving normal blood showed no such rise, and we are unable at present to offer any explanation. Text-fig. 3 graphically represents the typical temperature and leucocytic changes induced.



TEXT-FIG. 3. Temperature and leucocytic curves after inoculation of human measles blood secured at height of eruption.

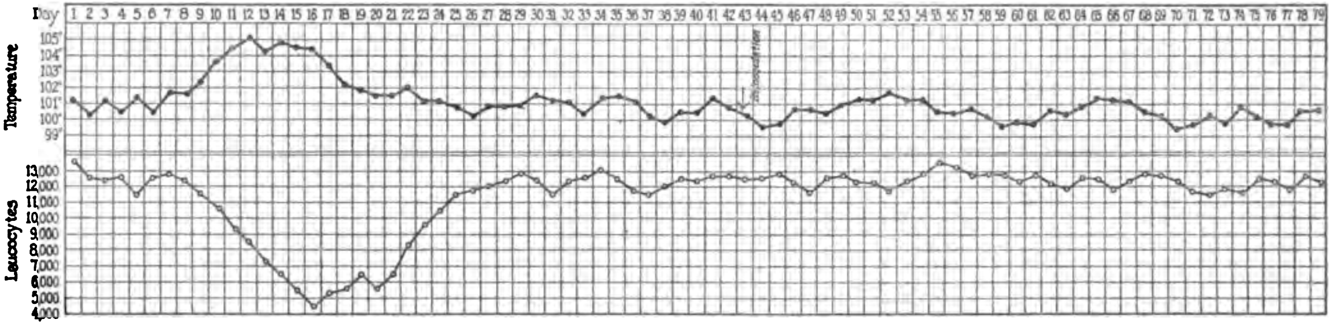
In order to test the effects of reinoculation upon previously reacting animals six recovered guinea pigs were reinoculated with 2 cc. portions of blood secured from human cases of measles at the height of eruption; in none were further pyrexial or leucocytic changes noted (Text-fig. 4).

A number of these animals were sacrificed when the reaction was at its height, and at autopsy the kidney invariably presented microscopic and often gross evidence of acute hemorrhagic nephritis. Blood was removed from certain animals of the series without killing them, and transferred to new guinea pigs for the purpose of virus propagation. Cultural control of the withdrawn blood, which was always carried out, proved negative in all cases for ordinary microorganisms.

Experiment 4.—The purpose was to continue the propagation of the virus by passage from guinea pig to guinea pig. Blood of animals reacting to the direct transfer of measles virus from man was removed by cardiopuncture at the height of the reaction, defibrinated, and injected in 1 cc. quantities directly into the heart chamber of new animals. Seven different passage strains were used and four to six guinea pigs employed. With two strains the transfers were discontinued after the third generation, while with four others the passage was carried to the eighth generation. In these animals the proportion of reactions was greater than with those of Experiment 3. In some series all the guinea pigs reacted in a characteristic manner, in others only part. The reaction was identical with that observed in the direct transmission experiments. After an incubation period of 9 to 10 days there occurred a rather abrupt rise in temperature of 2–4°F. and a coincident fall in the leucocytes. The temperature remained at the peak for 2 to 3 days, gradually subsiding, and reaching normal on the 14th to 16th days after inoculation.

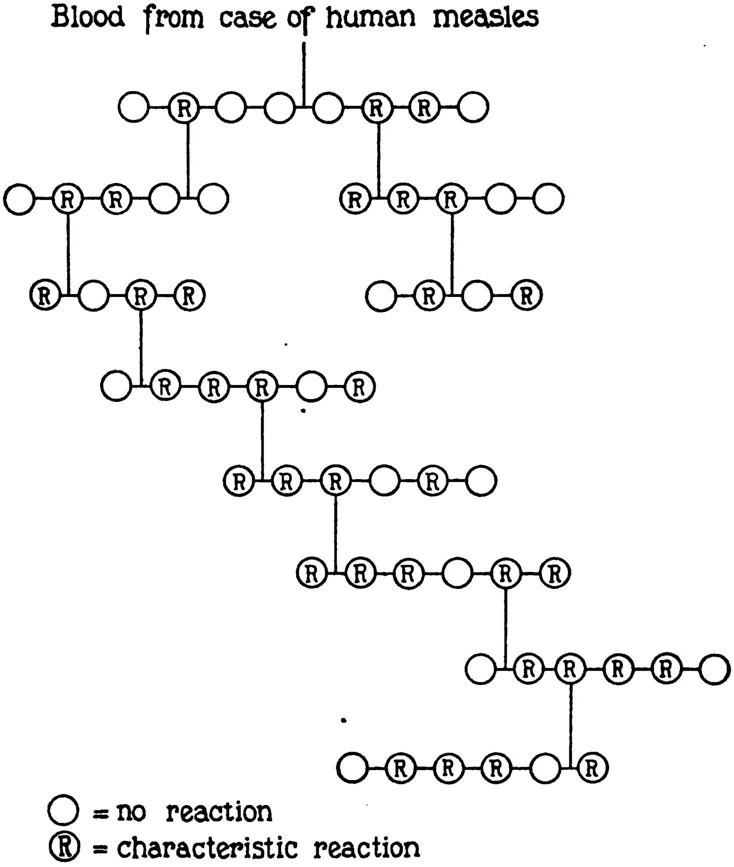
In this experiment it is noteworthy that a number of guinea pigs succumbed presumably as a result of the inoculated virus. Death occurred not infrequently in virus animals of the third generation. Such animals were visibly sick during the period when the reaction was at its maximum; namely, on the 9th to 14th days. They became listless, the hair ruffled, and they remained in the corner of the cage refusing food. Autopsies showed an acute hemorrhagic nephritis which was confirmed by microscopic study. Cultures from the heart's blood, kidney, etc., were always negative. Aside from acute congestion and slight parenchymatous changes, other organs showed nothing worthy of note. The lungs in no instance revealed lesions of any significance. Text-fig. 5 expresses graphically the propagation experiments with a typically reacting passage strain.

Experiment 5.—Since we found that the guinea pig reacted specifically to the intracardiac injections of measles virus, three series of experiments were carried out with a view of ascertaining the relative period of infectivity of the human blood containing virus for the guinea pig. One series of tests comprised twenty-four guinea pigs which were inoculated by the cardiac route with 1 cc. of fresh defibrinated blood that had been secured 1 hour previously from four children exposed to measles by living in close contact with members of their family suffering from



TEXT-FIG. 4. Effects of reinoculation with human defibrinated measles blood secured at height of eruption on temperature and leucocytes of guinea pig which had reacted previously.

the disease. All these children showed 0.5°–1° rise in temperature and some malaise, and two showed slight coryza. Clinically they were classed in the prodromal stage; later all these children developed measles. Subsequently it turned out that the blood was secured 60, 45, 40, and 26 hours, respectively, before the appearance of the eruption. A second series of twenty-four guinea pigs was injected intracardially with 1 cc. of defibrinated blood from four cases of measles in the preeruptive stage. These cases at the time of collecting the blood showed temperatures ranging between 99° and 100°F. and marked coryza, and two showed Kopliks spots. In this experiment the blood injected was withdrawn from the patients 36, 30, 16, and 12 hours previous to the appearance of



TEXT-FIG. 5. Showing the continued reaction in guinea pigs through eight successive generations for a single strain of measles virus.

the rash. The third series comprised eighteen guinea pigs which received 1 cc. of fresh defibrinated measles blood secured at periods from 24 to 48 hours after the full abatement of clinical symptoms. It is noteworthy that one of the bloods used was obtained from a case previously tested during the eruptive stage, and from which animal inoculations gave typical leucocytic and temperature reactions.

Animals of Series 1, which were to test the infectivity of the blood in the prodromal stage of measles, did not show at any time an elevation in temperature or decrease in the leucocytes though observed daily over a period of 5 weeks. The negative result in all these animals would indicate that the blood of cases of human measles is not infectious for the guinea pig during the so called prodromal period. As further indication that such is the case, some of these animals were subsequently inoculated with 1 cc. of blood containing virus secured during the eruptive stage, with resulting typical reactions. It is possible, though not probable, that the failure of the animals to respond was due to the small amount of the specific excitant which existed in the quantity of blood injected. We are inclined to believe, however, that the virus had not yet invaded the blood at this stage.

With the second series of animals, the purpose of which was to determine the infectivity for the preeruptive stage, very few reacted, and in none were the reactions very definite. In three out of a total of twenty-four there occurred a slight rise in temperature after an incubation period of apparently 2 weeks, but at no time was there an appreciable decrease in the leucocytes. Two of these animals, however, subsequently failed to react when injected with 2 cc. of blood secured from a human case at the height of eruption. This might be considered as acquired protection in the guinea pig produced by a previous infection. At any rate we conclude from this experiment that the blood of preeruptive cases of measles is not infectious for the guinea pig beyond 12 hours preceding the eruption.

In the third series, in which it was attempted to determine how long the blood of the convalescing human case remains infectious for the guinea pig, none of the eighteen animals inoculated with blood collected 24 hours after the temperature had reached normal, even though the rash was still discernible on the patient, reacted in a suggestive manner.

It would then seem that the infectious phase of the human, virus-containing blood for the guinea pig has a range of approximately 54 hours. This time includes in man the latter part of the preeruptive, and the early part of the temperature declining periods, in addition to the period of temperature height and full eruption.

Experiment 6 (Cultural).—We desire at this time to record very briefly the cultural experiments which were performed during the early part of this study. Blood from cases of human measles and from guinea pigs which had previously reacted to inoculation was defibrinated and cultured on a variety of media. For instance, aerobic and anaerobic cultures were made with red agar, chocolate agar, potato blood agar, and Noguchi media both solid and fluid, as substrate. Quantities of blood varying from 0.1 to 1 cc. were used as the inoculating material, with incubation at various temperatures for periods ranging from 1 to 25 days. No macroscopic or microscopic growths could be discerned. On a number of occasions we encountered peculiar structures resembling somewhat in appearance the globoid bodies of Flexner and Noguchi.[12] We mention this purely for the sake of completeness, as for the present we are entirely disregarding them and are inclined to look upon them as colloidal aggregates.

SUMMARY.

In general it may be said that three striking alterations occurred constantly in the animals reacting to intracardiac injections of blood from cases of measles; namely, pyrexia, leucopenia, and nephritis. The elevation in temperature usually began about the 9th day following inoculation, the rise being fairly abrupt from the normal to 104°F. and above, at which height it remained with slight fluctuations for 3 or 4 days, finally subsiding by lysis. During the period of fever, particularly in the animals of first transmission, often no objective signs of illness were to be noted, the guinea pigs remaining lively and eating as usual. When the animals showed any outward signs these were manifested by a loss of appetite, lusterless and ruffled hair, and indisposition to move even when disturbed. The typical exanthematous signs of human measles were not observed in any of the animals. In some there occurred a slight coryza and watering at the eyes; however, no special account was attached to these signs as they were rather indefinite and inconstant, especially for animals of first transmission.

Coincident with the rise of temperature there always appeared a fall in the total leucocytic count; in some animals the drop in white cells preceded the rise in temperature by 2 to 3 days. The leucopenia, though well defined, varied for animals of the same series. In some the count was as low as 3,600 cells per c. mm.; in no case did we fail to note at least a moderate fall in the leucocytes. The average time for the appearance of this cellular change was 9 to 10 days following inoculation; in other words, the leucocytic reaction seemed to indicate the end of the incubation period. So constant and striking was the leucopenia that we stress this as the most significant feature of the reaction of the guinea pig to the injection of measles blood, particularly since normal blood produced not the slightest change in these white elements. The greatest leucocytic depression was observed around the 12th to 14th days, which corresponded approximately to the stage of temperature peak. Following the leucopenia, the leucocytic rise was very gradual, taking on the average 8 days to attain the normal level.

---

[12] Flexner, S., and Noguchi, H., Experiments on the cultivation of the microörganism causing epidemic poliomyelitis, *J. Exp. Med.*, 1913, xviii, 461.

The animals killed at the height of the reaction, or those dying presumably from the effects of the blood containing virus, showed as the most constant lesion gross evidences of acute nephritis. The kidneys in these cases were swollen, cloudy, and congested. In certain guinea pigs there were in addition well defined petechiæ and larger blood extravasations scattered throughout the cortical substance. In the animals dying after inoculation, the kidney alterations were found so constant and characteristic that we regarded them as a special index of the experimental infection. In these animals the hemorrhagic areas were not infrequently 2 to 5 mm. in extent, and when occurring on the surface of the organ produced a separation of the capsule. Occasionally free blood was noted in the pelvis, and in the absence of hemorrhage within the pelvic lining, we assumed that it came from the uriniferous orifices of the calices. Microscopically the kidney sections revealed evidence of acute hemorrhagic nephritis, the hemorrhages for the most part being related to the capillaries of the tufts and the tubules of the pyramids. In the most pronounced cases practically all glomeruli were affected. Every stage from marked dilatation of the capillary whirl to well defined intercapillary blood extravasations that often filled the capsular space, and in consequence partially or completely obliterated the glomeruli, were found. Whether a destruction of the endothelium occurred, could not be determined. The absence of a neutrophilic reaction was of special significance, these elements of acute inflammation being nowhere found associated with the kidney lesion. The blood in relation to the tubules commonly occurred in the lumen of the collecting tubules and in the form of red blood cell casts.

The spleen usually was found enlarged, particularly in the guinea pigs presumably dying as a result of the virus injections. Aside from the usual parenchymatous changes common to toxemias, the other internal organs showed nothing of special note.

CONCLUSIONS.

Guinea pigs react specifically to intracardiac injections of defibrinated blood from cases of human measles.

There is a definite and constant rise in the temperature and a coincident decrease in the total number of leucocytes after an incubation period of 9 to 12 days.

The guinea pig reaction is produced with the human blood only during a certain phase of the disease, which period corresponds approximately to the eruptive stage. 36 hours prior to the eruption and 24 hours after the temperature is normal, the human blood gives rise to no reaction in this animal. The reaction follows with greater frequency in animals inoculated with measles blood obtained at the height of the eruption.

Guinea pigs which react and recover are not susceptible to reinoculation with measles blood if tested over periods of 2 weeks to 3 months after recovery.

Guinea pigs receiving normal human blood injected intracardially do not react with leucocytic or temperature changes.

The constancy of the reaction described leads us to conclude that propagation of the virus is obtained by passage of the blood from infected guinea pig to normal guinea pig; that such passage seemingly increases virulence, since a number of animals thus inoculated die during or subsequently to the peak of the reaction. Apparently death in these cases results from acute hemorrhagic nephritis. Ordinary intercurrent or secondary infection plays no part in these effects as shown by careful bacteriological examination.

Postmortem examinations of animals sacrificed at the height of reaction, and of animals dying, presumably, as the result of the specific infection, show an intense hemorrhagic nephritis, which is a constant pathological finding.

Attempts to cultivate the virus of measles so far have been unsuccessful.

A typical exanthem is not noted in the guinea pig following the intracardiac method of inoculation of human blood containing virus. Coryza and injection of buccal mucous membrane occur in some animals, but are so inconstant as not to be included by us as part of the specific reaction.

While the symptoms and lesions produced in the guinea pig are not entirely analogous to those in man, the constancy of a characteristic reaction after a definite incubation period, together with the successful reproduction of this reaction in subsequent guinea pig to guinea pig inoculations, indicates that the virus of measles is capable of being propagated in the guinea pig, and is pathogenic for this animal.

HEMOLYSIS OF ERYTHROCYTES IN CONTACT WITH GLASS.

BY WALLACE O. FENN, PH.D.

(*From the Laboratory of Applied Physiology of Harvard Medical School, Boston.*)

(Received for publication, September 24, 1921.)

In the course of experiments in which it became necessary to make counts of the numbers of red blood corpuscles in various solutions without the use of fixatives it was observed that the cells began to hemolyze as soon as they settled out in the hemocytometer, even though the same cells might remain intact for hours when not in contact with the glass. Hemolysis occurred in some cases so rapidly that it was impossible to make accurate counts. Hemolysis of this sort must have been frequently observed by others, but the literature seems to contain no mention of the fact, nor does any study seem to have been made of the conditions underlying the phenomenon. An investigation of the subject was undertaken with the idea that it might throw some light upon other well known reactions between blood cells and solid bodies, such as the phagocytosis of solid particles, the spreading of leucocytes against solid surfaces, and the disintegration of blood platelets upon contact with foreign bodies.

EXPERIMENTAL.

Contact Hemolysis.

The phenomenon of contact hemolysis may be easily demonstrated in the following manner. A drop of blood is taken from the finger into 25 cc. of 0.9 per cent sodium chloride. The cells are centrifuged out and resuspended in 25 cc. of 0.9 per cent sodium chloride. If a drop of this suspension is mounted on a slide which is not scrupulously clean, the more or less rapid hemolysis of the cells may be readily observed, especially if no precaution has been taken to neutralize the sodium chloride solution which, ordinarily, is as acid as pH 5, even when prepared from chemically pure material. Under favorable circumstances all the cells may have disappeared in 5 minutes, although there has been no change in the original material. Quantitative data are obtained in the same manner by making frequent counts of the numbers of corpuscles still intact on the slide and in suspension.

TABLE I.

Time Required for 50 Per Cent Hemolysis of Washed Erythrocytes Resting on Glass and in Suspension.

Erythrocytes.	pH	Time required for 50 per cent hemolysis.		
		In suspension.	On glass.	
		hrs.	*hrs.*	*per cent**
Human.	3.5	0.18	0.07	39
	4.0	0.27	0.17	63
	4.0	0.2–0.4	0.10	30±
	5.2	1–2.+	0.60	50–
	6.0	3.+	2.+	—
	9.3	2.90	1.10	38
	10.0	2.+	0.87	50–
Rabbit.	3.3	0.20	0.07	35
	3.5	0.32	0.07	22
	3.6	0.36	0.10	28
	3.8	0.52	0.05	10
	4.0	0.31	0.06	19
	4.2	0.6–1.0+	0.15	25–
	9.6	0.43	0.22	51
	9.7	0.32	0.22	69
	9.8	0.37	0.23	62
	9.8	0.30	0.24	80
	10.05	0.40	0.44	110
Average..				47

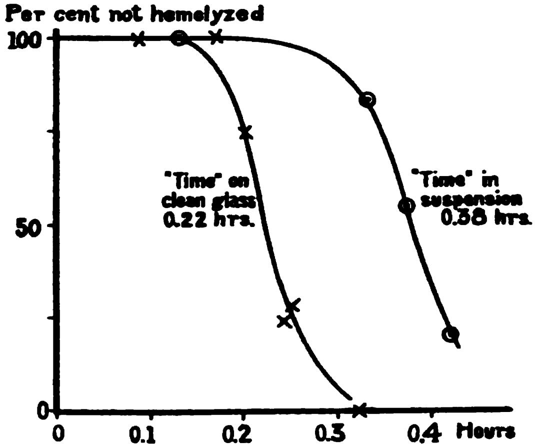
* Time on glass in per cent of time in suspension.

In some cases the hemolysis under the cover-slip was quite irregular in different parts. This is indicated in the tables either by two figures (as 0.26–6.0), showing the approximate extent of the irregularity, or by a minus sign, indicating that the counts were made where hemolysis was slowest.

The results of eighteen such experiments with washed rabbit and human erythrocytes are given in Table I. In using human blood, approximately 0.01 cc. was taken from a finger-tip in a calibrated capillary pipette, washed once in 25 cc. of 0.9 per cent sodium chloride, and resuspended in a similar volume of isotonic salt solution. Mixtures of varying pH were prepared by taking 5 cc. of this suspension (in some experiments only 1 cc.) plus 1 to 5 cc. of 0.004 M sodium hydroxide or 0.005 M hydrochloric acid in 0.9 per cent sodium chloride plus enough 0.9 per cent sodium chloride to make 15 cc. The pH was measured by a Bovie hydrogen ion potentiometer and a Clark hydrogen electrode. In experiments on rabbits, 0.3 cc. of blood was taken from the ear vein of a rabbit into a pipette previously rinsed in a 0.5 per cent solution of sodium oxalate in 0.9 per cent sodium chloride to delay clotting, centrifuged in 50 cc. of 0.9 per cent sodium chloride, and resuspended in a similar volume of salt solution. Mixtures were then prepared as for human blood, with the exception that usually only 1 cc. of cells was used.

To determine the rate of hemolysis on glass, a cover-slip was supported on the slide at four points by fragments of another cover-slip; and the mixture was allowed to run under the cover-glass, which was then sealed with paraffin. Counts were made at once over equal areas as measured by an ocular micrometer disc ruled in squares. Frequent counts were made during the experiment, the points on the slide counted being chosen more or less at random in order to average to some extent the variations sometimes observed even under the same cover-slip. The course of hemolysis in the remainder of the mixture, left in suspension in the test-tube, was followed simultaneously. It was thought advisable, for the sake of uniformity, to do this by counting the number of intact corpuscles in a given volume on a hemocytometer. Ordinarily about 200 cells were counted for each determination. The results were then calculated in per cent of the original, plotted on coordinate paper, and the time necessary for 50 per cent hemolysis was determined graphically. Text-fig 1. shows two typical curves having the characteristic S shape of hemolysis curves. The time is determined most accurately at the 50 per cent mark, the point of most rapid hemolysis.

For "clean glass" experiments the slides were first thoroughly washed by the laboratory assistant by the usual methods, including treatment with soap and water, cleaning solution, and finally drying with a clean towel. For uniformity they were again allowed to remain in cleaning solution for a time, were rinsed in hot running tap water for 10 to 20 minutes, then in boiling distilled water, and were finally stacked on a glass rack where they dried in less than a minute. During the final stages of the process they were handled only with glass tweezers. This method has the advantage of avoiding the grease which inevitably collects on the cleanest towel and it saves the labor of drying. It is easy to demonstrate the presence of what is probably a film of grease on a slide by scraping the surface gently with a pair of tweezers held perpendicularly. On a perfectly clean slide a distinct squeak is heard. On slides cleaned by the usual methods involving wiping, there is usually sufficient grease to act as a lubricant so that the tweezers move smoothly over the surface.



TEXT-FIG. 1. Curves showing the percentages of washed rabbit erythrocytes still intact at various times after mixing. The time necessary for 50 per cent hemolysis of these cells in suspension (pH 9.8) was 0.38 hours, while the hemolysis time for the same cells resting on glass was only 0.22 hours.

The figures in Table I show that hemolysis of washed human and rabbit erythrocytes which are resting on clean glass occurs in less than one-half the time necessary for the cells in suspension. That this cannot be due to small amounts of alkali is shown by the fact that the phenomenon occurs at the point of acid hemolysis as well as at the point of alkali hemolysis. It is, if anything, more pronounced in acid solutions.

Since it was most natural to connect this phenomenon with the clotting of blood on contact with foreign bodies and the disintegration of blood platelets, it seemed advisable to see whether the disintegration of the erythrocytes could be prevented by a thin film of vaseline, paraffin, or paraffin oil on the slide. In Table II the hemolysis time of washed human erythrocytes in suspension is compared with the hemolysis time on a slide covered with a thin film of vaseline. The vaseline was taken from a solution in ether used in the laboratory for coating cannulas to prevent clotting. As on clean glass, the erythrocytes hemolyze more than twice as rapidly on vaseline as they do in suspension. Many of the experiments on vaseline were done simultaneously with experiments on clean glass, with the same mixtures. In such instances, when a direct comparison between vaseline and glass was possible, no significant difference between the two was found. The film of vaseline was just visible to the naked eye; and the microscope revealed many small drops of vaseline, which rounded up in contact with the water and sometimes became hardly distinguishable from blood corpuscles.

TABLE II.

Time Required for 50 Per Cent Hemolysis of Human Erythrocytes on a Vaselined Slide and in Suspension.

pH	Time required for 50 per cent hemolysis.		
	In suspension.	On vaseline.	
	hrs.	*hrs.*	*per cent*
3.3	0.20	0.07	35
3.5	0.18	0.10	55
4.0	0.27	0.17	63
6.0	3.+	2.+	—
10.0	2.+	0.60	30−
10.3	1.+	0.37	37−

Table III gives the results of another experiment with washed rabbit corpuscles in which the time of hemolysis on paraffin and paraffin oil was compared with that in suspension. The paraffin oil ("highest purity"), like the vaseline, offers no protection against hemolysis. The results with paraffin are more irregular, but here too, in most cases, hemolysis is more rapid in the sedimented cells than in those suspended. There seems to be some other factor involved in the case of paraffin, however, and to some extent with paraffin oil, because the time curves of hemolysis have frequently an abnormal shape, indicating that hemolysis begins very rapidly and stops short of completion, leaving part of the cells intact, sometimes for hours, after all the suspended cells have disappeared. The reason for this abnormality is not apparent. The paraffin or paraffin oil was applied to the slide with a clean cloth, thus producing a thin film barely visible to the naked eye but very evident under the microscope—as ridges in the case of paraffin or as drops in the case of paraffin oil. A typical experiment of this series is plotted in Text-fig. 2.

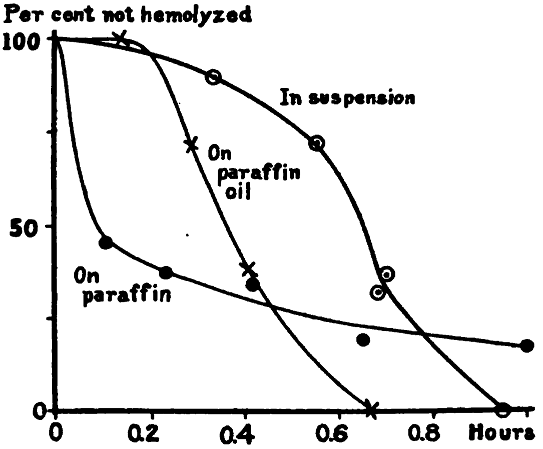
TABLE III.

Time Required for 50 Per Cent Hemolysis of Rabbit Erythrocytes on Films of Paraffin and Paraffin Oil, and in Suspension.

pH	Time required for 50 per cent hemolysis.				
	In suspension.	On paraffin.		On paraffin oil.	
	hrs.	*hrs.*	*per cent*	*hrs.*	*per cent*
3.3	0.20	0.14*	70	0.08*	40
4.0	0.44	0.26–6.0	59+	0.10	23
4.0	0.45	0.12	27	0.14	31
4.2	6.0	6.00	100	0.60	10
9.7	0.56	0.13	23	0.25–0.35	45–53
9.7	0.68	0.09–	13–	0.36–	53–
10.3	0.38	0.38	100	0.18–	47–
11.05	0.15	0.10	67	0.04	27
11.4	0.07	0.03–0.08	42+	0.05	70

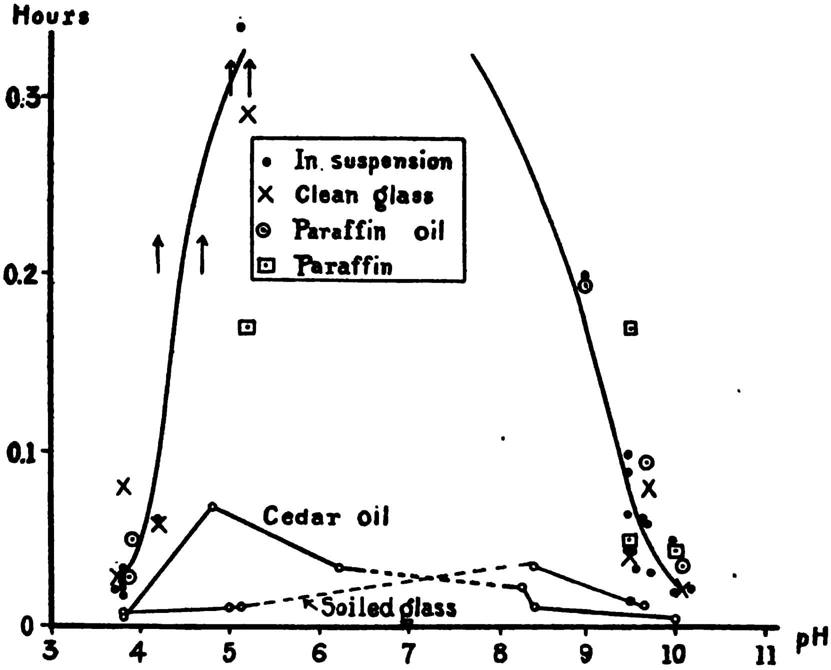
* Not completely hemolyzed until after the control, the shapes of the curves being different as explained in the text.

While contact hemolysis does occur with clean glass, it is much more pronounced with slightly soiled glass. Text-fig. 3 represents the results of a series of experiments in which the hemolysis of rabbit erythrocytes on paraffin, paraffin oil, clean glass, and in suspension is compared with the time necessary for hemolysis of the same cells in contact with a soiled slide. The slides were first washed clean by the usual method and were then wiped with a cloth which had been rubbed once on the hair but which was otherwise clean. This treatment produced a dimly visible film on the surface of the glass. The extremely rapid hemolysis is very evident from the figure. A similar experiment, with slides coated with a specimen of cedar oil used for immersion lenses also shows abnormally rapid hemolysis. There was no assurance of the purity of this oil, however, and the result is not of great significance. This figure shows that there is no constant difference between the rate of hemolysis in suspension and the rate of hemolysis on glass, paraffin, or paraffin oil. The apparent discrepancy between this experiment and those previously cited is due to the serum content of the mixture, since the cells were not washed before use. Allowing 40 per cent for the volume of the corpuscles in whole blood, it may be calculated that the serum content of the unwashed cells in the final mixture (5 cc. of cells to 10 cc. of hydrochloric acid or sodium hydroxide) was 0.12 per cent of the normal serum content of blood. Even with this small concentration of serum, contact hemolysis is prevented to a considerable extent on clean glass, though in some experiments it was still evident, particularly in acid solutions. The results of all available comparisons on



TEXT-FIG. 2. Curves showing the rate of hemolysis of a suspension of washed rabbit cells (2,200 per c. mm.) at pH 9.7 resting on films of paraffin and paraffin oil, and in suspension.

clean glass are given in Table IV. In not a few instances the cells on the glass hemolyze even less rapidly than those in suspension. Comparison with Table I reveals the striking effect of small amounts of serum in hindering contact hemolysis.



TEXT-FIG. 3. Curves showing the time necessary for 50 per cent hemolysis of rabbit erythrocytes in 0.12 per cent serum in solutions of various pH values. The rate of hemolysis in suspension is compared with the rate of hemolysis when the cells were resting on clean glass or glass covered with films of paraffin, paraffin oil, cedar oil, or dirt obtained by rubbing a clean cloth on the hair. Arrows indicate that the hemolysis time was greater than the value indicated. Figures showing the hemolysis time in suspension and on clean glass under these conditions are given in Table IV.

In some of the experiments with serum, unwashed cells were used; while in others an equivalent amount of serum was added to washed cells. No alteration in behavior due to the washing was detected.

Further experiments would be desirable in order to see whether the effect of serum is due to the calcium content and whether it can be destroyed by heating. One experiment was tried to make sure that it was not due to the small amount of oxalate present from rinsing the pipette before taking the blood, but no change in the rate of hemolysis was detected from twice the usual amount of oxalate in the same volume of sodium chloride without serum. In one experiment 0.02 per cent of serum was found to prevent hemolysis on glass almost completely, which otherwise took place in about half an hour. This was probably largely due to the buffer action of the serum. No measurements of the pH were made in this experiment.

TABLE IV.

Time Required for 50 Per Cent Hemolysis of Rabbit Erythrocytes in 0.12 Per Cent Serum on Clean Glass and in Suspension.

pH	Time required for 50 per cent hemolysis.		
	In suspension.	On glass.	
	hrs.	*hrs.*	*per cent*
3.3	0.24	0.08	33
3.3	0.23	0.15	65
3.4	0.23	0.15	65
3.8	0.40	0.27	68
3.8	0.35	0.27	77
3.8	0.22	0.5–1.0	228+
3.8	0.28	0.28	100
3.8	0.25	0.22	88
4.0	0.17	0.26	153
4.0	0.18	0.22	120
4.0	0.22	0.27	122
4.2	2.+	2.+	—
4.2	0.64	0.6	94
4.7	2.+	0.8–1.2	50–
5.2	3.+	2.8	90–
6.4	1.+	1.+	—
9.5	0.44	0.43	98
9.7	0.54	0.80	140
9.7	0.42	0.42	100
9.8	0.36	0.36	100
9.9	0.24	0.23	96
10.0	0.35	0.41	117
10.2	0.20	0.25	125

In a few preliminary experiments it was found that 2 per cent dextrose and 0.5 per cent acacia definitely inhibited hemolysis of human red cells at pH 5.2, while 0.25 per cent acacia failed to do so. More determinations are necessary to establish this as a fact. Interest in these substances arises from the work of Rous and Turner (1), who recommend dextrose-Locke's solution for preserving red cells and gelatin to protect them from injury during centrifuging. Acacia might be expected to resemble gelatin in this respect.

In view of the hemolyzing effect of small amounts of impurities on the glass it is evident that the interpretation of the results is always open to the possible but improbable objection that the glass was not perfectly clean. It was not thought practicable, however, as a routine measure in such a large number of determinations to subject the glass to more extended treatment, such as rubbing with grease-free diatomaceous earth, etc. It is evident, nevertheless, that contact hemolysis is a phenomenon which must occur frequently in experiments on the preservation of red blood corpuscles *in vitro* (1). Also, the hemolysis often observed in centrifuging washed red blood corpuscles is doubtless of a similar nature. In all such experiments it is essential that the pH be controlled more accurately than has usually been considered necessary, since the hemolysis limits are much narrower for cells in contact with glass. The usual acidity of chemically pure preparations of sodium chloride (pH 5.0) and the alkalinity of sodium citrate solutions make this particularly advisable.

A number of experiments were tried with fresh mica surfaces, in the hope that they might be found cleaner than paraffin or glass, but the result was even more irregular. In some areas on the slide the cells hemolyzed much more rapidly, in others somewhat less rapidly than the control cells in suspension. On the whole, it may be said that contact hemolysis occurs on mica as well as on the other surfaces used.

In explaining these results three possibilities are open. In the first place, one thinks of soluble materials coming from the glass. Thus Hausmann and Kerl (2), in studying oligodynamic hemolysis by various metallic plates, observed that hemolysis occurred also around a glass rod left in the plate of agar containing the red blood corpuscles, and attributed the results to alkali given off by the glass. This explanation, however, seems excluded here because of the fact that contact hemolysis occurs in acid as well as in alkaline solutions.

Another proposal is that of Schwyzer (3), who believed that the formation of rolls by red blood corpuscles in contact with glass was due to a neutralization of their electric charge. This explanation is also somewhat improbable in view of the fact that contact hemolysis occurs on both sides of the isoelectric point of the red blood corpuscles (pH 4.7, as given by Coulter (4)).

A more useful working hypothesis seems to be that contact hemolysis is the result of an attempt on the part of the corpuscle or, perhaps, some ingredient of the corpuscles, as the lipoids, to spread on the glass by surface tension, thus accelerating the disintegration of the structure. It may be supposed that this spreading would occur in neutral as well as in acid and alkaline solutions if it were not for the more or less rigid structure of the corpuscle.[1] This conception is readily correlated with the experiments of Bechhold (5), who observed that when red blood corpuscles were shaken with clay or kieselguhr, the particles of the latter could be observed sticking to the outside of the cells. On shaking the mixture vigorously enough to detach these particles, hemolysis occurred. His interpretation was that the particles tore away with them parts of the cell (he suggests lipoid), thus serving to break up the intimate association of lipoid and protein upon which he conceives the rigidity of the corpuscle to depend. The rigidity of the cells, which keeps them disc-shaped, is obviously reduced in alkaline solutions when they round up. During acid hemolysis on the glass the cells are larger in diameter, either because of swelling (Price-Jones (6)) or because they spread out on the glass into a hemispherical shape. Simultaneously they become lighter in color and focus the rays of light at a higher point than do normal cells. This may be due to a more or less hemispherical shape (such as obtains in a cell which spreads out), or to a lower refractive index, or to both.

If this hypothesis were true, the erythrocytes might be expected to stick to the glass most firmly in those solutions in which they show contact hemolysis to the greatest extent. From a few experiments on the stickiness of erythrocytes, which are described later, it may be concluded that the cells are more sticky in acid solutions and less so in alkaline than they are at neutrality. This observation clearly supports the idea that contact hemolysis, in the acid range at least, is due to a spreading out of the cell. If the same conception is to be applied to alkaline solutions in which hemolyzing cells are, if anything, smaller than normal and in which their stickiness is also less, it must be assumed that some ingredient of the cells, as the lipoids, is spreading invisibly. Since there is as yet no experimental basis for this idea, speculation is futile. It may be pointed out, however, that the flotation processes for the separation of different ores depend in general upon the greater tendency of certain particles of ore to stick to oil drops, suggesting by analogy a greater attraction of the glass for lipoids than for other parts of the cell. However that may be, some readjustment of the cellular equilibrium would be expected, from ordinary principles of surface tension, when a complex system of different phases like a red blood corpuscle comes into contact with a solid body. While this readjustment may not have a detectable effect on a normal cell, it may well accelerate disintegration of a cell which is already at the point of acid or alkaline hemolysis.

---

[1] This seems somewhat more probable than the alternative assumption that the cells are not pulled out by surface tension at neutrality as strongly as in acid or alkaline solutions.

It is possible that differences in the sensitivity of erythrocytes to contact with glass may occur between species or between individuals, though no attempts have been made to detect them. The method, however, is not very well suited for the measurement of small variations. One is always confronted by the possibility of invisible films of impurities on the glass which destroy the constancy of the results.

Experiments on Stickiness of Erythrocytes.

Experiments on the stickiness of erythrocytes were undertaken by two methods. In the first, the erythrocytes were allowed to settle out in a vessel made by sealing, with paraffin, a short glass cylinder, 2 cm. in diameter and 3 cm. long, to a microscope slide, the other end being closed by a rubber stopper with a capillary air vent. After an hour or two the vessel containing the supernatant solution and the sedimented cells was rotated on a motor ten or fifteen times in as many seconds about a horizontal axis, thus permitting the circulating liquid to wash off a certain proportion of the cells. This proportion was determined by counting the cells washed off and those still sticking on in known volumes and areas respectively.

The effect of pH on the stickiness of washed rat erythrocytes as measured by this method in two experiments is shown by the following figures.

pH	Erythrocytes stuck on slide.	
	A	B
	per cent	*per cent*
5	17	15
7	0	9
9	0	0

The H ion concentration was maintained by buffer solutions of phosphates in $\frac{M}{60}$ solutions. At pH 5, in both cases, many hemolyzed cells, which had not been washed off when the vessel was rotated, could be dimly seen on the slide. This shows that after contact hemolysis in acid solutions the remains of the cells are stuck to the slide and indicates that contact hemolysis in acid solutions may be due to a spreading of the cells.

In another similar experiment, in which there was apparently no hemolysis, no cells were found sticking to the slide at pH 7 or 9, while there were 700 per sq. mm. at pH 5.

Further measurements by the same method with varying concentrations of serum indicate again an apparent relation between contact hemolysis and stickiness. The results were as follows:

Serum.	Erythrocytes.		
	Washed off.	Stuck on slide.	Remainder, hemolyzed.
per cent	*per cent*	*per cent*	*per cent*
0.00	43 ± 9	10	47
0.17	100 ± 6	0	0
0.35	83 ± 7	0	17
0.70	85 ± 7	0	15

In the presence of serum, no cells stick to the glass and there is no appreciable hemolysis (0, 17, and 15 per cent). Without serum, 57 per cent of the cells are either stuck to the slide or hemolyzed, hemolysis being probably the end-result of sticking. The solution of 0.9 per cent sodium chloride, used to dilute the serum, was carefully neutralized. Hemolysis was doubtless accelerated by contact with the glass, which lasted in this instance for 1½ hours at 25°C.

The other method by which the stickiness of erythrocytes was measured consisted in allowing a suspension of red blood corpuscles in the solution under investigation to run under a cover-slip, which was supported at four points on a microscope slide by small fragments of a cover-slip, as in the experiments on contact hemolysis. The edges of the cover were sealed with paraffin. After a certain interval of time (5 to 15 minutes) the preparation was inverted and the percentage of cells which were stuck to the slide firmly enough to prevent them from falling off was determined by counting the numbers of cells observed over equal areas on cover and slide. In acid solutions almost no cells drop off no matter how long a time is allowed.[2] In alkaline solutions somewhat variable results are obtained, depending upon the time intervals allowed before and after inverting the preparation. This variation is probably due to the fact that it takes some time to reach equilibrium in alkaline solutions. If the suspension is mounted as soon as mixed and inverted as soon as the cells have settled out, it may take 15 minutes or an hour for the cells to drop off. If, however, 5 minutes are allowed between the time of mixing and the time of mounting, or if 15 minutes are allowed between the time of mounting and the time of inverting, a large proportion of the cells usually drops off during the first 5 minutes after inverting. Two series of experiments by this method are summarized in Table V. In Experiment A, the cells were left in suspension 5 minutes after mixing, to reach equilibrium, and the counts were then made within 5 minutes after inverting. In Experiment B, the cells were mounted immediately after mixing and inverted within 5 minutes, but counts were not made for 15 minutes after inverting. To avoid further complications, this study was limited to those cases in which hemolysis had not begun during the time of the observations. The pH values were determined electrometrically and were varied by the addition of 0.005 M solutions of sodium hydroxide and hydrochloric acid in 0.9 per cent sodium chloride. The cells were obtained by centrifuging a few drops of blood from a pricked finger in a known volume of salt solution. When the cells were not washed, the concentration of serum present was calculated from the number of erythrocytes present as determined by the hemocytometer, allowing 40 per cent for the volume of the corpuscles in fresh blood. The results obtained by this method as given in Table V confirm those obtained by the previous method, showing that human erythrocytes are more sticky in acid solutions.

---

[2] The behavior of erythrocytes in this respect reminds one strikingly of the device used by Perrin in determining Avogadro's constant by observations on a gamboge emulsion. Acidification of the solutions caused the globules of gamboge to stick to the walls of the vessel, where they could be easily counted (Perrin, J., Die Atome, Dresden, 1914).

TABLE V.

Effect of pH on the Percentage of Erythrocytes Sticking to Glass.

Experiment A.		Experiment B.	
pH	Erythrocytes stuck on slide.	pH	Erythrocytes stuck on slide.
	per cent		*per cent*
4.1	100	5.5	79
5.2	92	6.5	62
5.6	80	8.0	13
8.0	20	8.8	5
10.0	29	9.7	11
10.1	2		

Experiment A.—Cells in 0.05 per cent serum. They were allowed to run under a cover-slip on a microscope slide 5 minutes after adding sodium hydroxide or hydrochloric acid. 5 minutes later the slide was inverted. After another 5 minutes the percentage sticking on the slide was determined by counting.

Experiment B.—Cells in 0.9 per cent sodium chloride, no serum. They were mounted on the slide immediately after mixing, and counts were not made until 15 minutes after inverting. In alkaline solutions many drop off during this longer interval unless they are allowed time to come to equilibrium after mixing and before mounting. Variations less than 10 per cent are not significant.

SUMMARY.

1. Washed erythrocytes, in both acid and alkaline solutions, hemolyze more rapidly when allowed to settle out on a clean microscope slide than when kept in suspension.

2. This is also true on slides coated with paraffin, paraffin oil, or vaseline, and on mica surfaces.
3. The presence of as little as 0.1 per cent serum inhibits such contact hemolysis, particularly in alkaline solutions.
4. Contact hemolysis is most marked on slightly soiled glass, and may occur so rapidly with unfixed cells in a hemocytometer that accurate counts are rendered impossible.
5. Erythrocytes are more sticky than normally in acid solutions and less sticky in alkaline solutions.
6. The increased stickiness of erythrocytes in acid solutions and their larger size during contact hemolysis in acid media provide some experimental evidence for the view that contact hemolysis is to be correlated with an attempt on the part of the corpuscles, or some ingredient of the corpuscle, to spread on the glass after the manner of leucocytes and invertebrate blood cells.

BIBLIOGRAPHY.

1. Rous, P., and Turner, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 122.
2. Hausmann, W., and Kerl, W., *Biochem. Z.*, 1920, cxii, 122.
3. Schwyzer, F., *Biochem. Z.*, 1914, lx, 297.
4. Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.
5. Bechhold, H., *Münch. med. Woch.*, 1921, lxviii, 127.
6. Price-Jones, C., *J. Path. and Bact.*, 1920, xxiii, 371.

THE TWORT-D'HÉRELLE PHENOMENON.

II. LYSIS AND MICROBIC VARIATION.

BY ANDRÉ GRATIA, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 7 AND 8.

(Received for publication, June 28, 1921.)

In a preceding paper (1) the present knowledge of the phenomenon of transmissible microbic lysis discovered by Twort and by d'Hérelle was reviewed; there was described the method by means of which we dissociated a culture into two types of organisms, by merely allowing a culture of *Bacillus coli* to age. One, Type S, is very sensitive to destructive influences, such as desiccation and transmissible lysis. The other, on the contrary, is much more resistant. Besides these differences in vulnerability, the organisms are also distinguished by other properties; namely, motility and virulence.

Since that time we have had an opportunity to isolate a greater number of different types of *Bacillus coli*, all derived from the same original strain, as will be described in this article.

Characteristics of the Original Bacillus coli (coli O).

The culture employed was derived from a strain given by d'Hérelle to Bordet and Ciuca. Before beginning their experiment, Bordet and Ciuca made three successive isolations in order to start, as far as possible, with a culture arising from a single organism, and it was from this source that our culture was derived.

Besides the general properties of *Bacillus coli*, such as sugar fermentation and gas and indole production, this original strain of *Bacillus coli* possesses the following characteristics. The colonies on agar plates are large, flat, grape leaf-shaped, and very fluorescent. *Coli* O is non-motile and is very sensitive to the lytic agent, as is shown by the following tests.

Motility Test.—In order to test the motility of a strain, a stab culture is made into semisolid agar (0.5 per cent agar). If none of the organisms of a culture are motile, the growth is confined to the line of puncture. On the other hand, if they are all uniformly motile, they diffuse through the mass of agar and produce a uniform turbidity. If they have a variable motility the result is a combination of the two preceding pictures, that is to say, a dense growth appears close to the line of puncture and is surrounded by a diffuse zone of turbidity. This test is always controlled by a microscopic examination of broth cultures.

Resistance Test.—A suitable resistance test is found in the method of Bordet and Ciuca, slightly modified. These authors place a drop of lytic agent on a 3 hour culture of *B. coli* on agar slant. Resistant colonies grow on the path of clarification left by the drop. The number of these resistant colonies varies directly with the resistance of the strain.

In order to avoid the confusion that might result from the purely mechanical effect of the drop on the young growth, we prefer to place the drop on a sterile agar slant first, and then to seed the tube with *B. coli* 2 hours later. This method shows that *coli* O is very sensitive. Only very few or no resistant colonies appear in the zone of agar touched by the lytic agent.

Action of the Lytic Agent on coli O.—Bordet and Ciuca have shown that when a few drops of lytic agent are added to a broth culture of *coli* O, an almost complete dissolution occurs, with the exception of a very few resistant organisms. When transplanted on an agar slant these organisms produce a very scanty, irregular culture at the beginning, but after several passages the growth becomes more and more luxuriant and is distinguished from the original culture by certain characteristics. These organisms resist the lytic agent but have now acquired themselves lytic properties and become lysogenic, or capable of inducing dissolution in a culture of normal *Bacillus coli.* Moreover, when planted on slanted agar, a mucoid culture results. They are also less phagocytable and more virulent.

We found that all of the bacilli comprising a culture of this modified *coli* do not possess all of these characteristics, which are rather shared by different types of organisms in the culture. The following experiment shows that the modified *coli* is a very heterogeneous culture.

Experiment 1.—0.5 cc. of increasing dilutions of lytic agent (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, straight broth) were mixed respectively with 0.5 cc. of a 12 hour broth culture of *coli* O diluted to 10^{-5}. Immediately afterward, these mixtures were plated in Petri dishes with 10 cc. of plain agar and incubated at 37°C. After 12 hours the result shown in Fig. 1 was obtained; Plate A is the control, *e. g.* a normal culture of *coli* O, and the following plates, cultures of *coli* O mixed with an increasing quantity of lytic agent.

As shown in a preceding paper (1), the number of resistant colonies left varies in inverse proportion to the amount of lytic agent. But aside from this fact, it is possible to distinguish three types of colonies: (1) mucoid (Fig. 2); (2) non-mucoid and regular (Fig. 1, E and F); and (3) non-mucoid and irregular (Fig. 1, C and D).

Mucoid Colonies, or coli O R 1.

Such colonies are found only occasionally and it may be necessary to repeat the experiment several times to find one colony of this type. They are regular, large, convex, translucent, and non-fluorescent.

If a trace of one of these colonies is streaked on an agar plate, a curious picture results. The mucoid and translucent growth is studded with numerous opaque spots (Fig. 3, B). Among the isolated colonies some are entirely opaque, some entirely translucent, and some mixed. Transplanted, an opaque colony gives only a homogeneous opaque growth. On the contrary, a translucent colony reproduces the same mixture of translucent and opaque colonies. Thus, the translucent growth is a very unstable form which in an ordinary culture is very quickly overgrown by the more stable opaque form. In order to avoid complications, only the latter form will be considered under the distinctive denomination of *coli* O R 1.

Coli O R 1, like the original *coli* O from which it is derived, is non-motile but is completely resistant to the lytic agent. As is shown by the following test, it is not lysogenic or capable of inducing dissolution in a normal culture of sensitive *Bacillus coli.*

Lysogenic Test.—The technique is based on the fact that lysogenic organisms added, even in a very small number, to a young broth culture of sensitive *B. coli* produce lysis of this culture. The organisms to be tested are planted in 5 cc. of broth (pH 8.0). When the culture is 18 hours old, it is centrifuged and 0.5 cc. of the clear supernatant fluid, still containing a small number of organisms, is added to 5 cc. of a young broth culture (3 hours, pH 8.0) of normal *coli* O. When the growth of the mixture, compared with a normal culture of *coli* O as control, exhibits evidence of inhibition and dissolution, the test is positive.

This is not the case for *coli* O R 1.

To summarize, *coli* O R 1 is a non-motile, very resistant, and non-lysogenic organism producing large, round, mucoid, and non-fluorescent colonies.

Non-Mucoid Regular Colonies, or coli O R 2.

These colonies are found in the two last plates of Experiment 1; that is to say, in those plates in which the lytic agent is present in high concentration (Fig. 1, E and F). They are small, round, convex, hyaline, and non-fluorescent and retain these characteristics when transplanted on agar plates (Fig. 3, A). *Coli* O R 2 also is very resistant, non-lysogenic, and non-motile.

Non-Mucoid Irregular Colonies.

They exist only in the plates in which the lytic agent is present in moderate quantity (Fig. 1, Plates C and D). They are large, flat, non-fluorescent, and extremely irregular.

Experiment 2.—A trace of an irregular colony was streaked on an agar plate. In the first streaks the growth was scanty and irregular; but in the subsequent streaks the colonies became less irregular, showing sometimes only a slight indentation confined to a portion of the edge, and finally, perfectly regular large flat colonies could be found in the last streaks.

Irregular colonies can be transplanted in series many times with the same result. Nevertheless, after a certain number of generations, the proportion of regular colonies appearing in the last streaks increases.

Experiment 3.—An irregular colony, or the irregular edge of a partially irregular colony, was planted in broth. A very slowly growing culture resulted which was lysogenic.

Irregular colonies are thus lysogenic. On the other hand, the regular colonies found in the last streaks have lost this property, as is seen in the following experiment.

Experiment 4.—A regular colony, or the regular edge of a partially irregular colony, was streaked on an agar plate. Only regular colonies appeared. They were large, flat, and non-fluorescent. Transplanted in broth, they gave a normal rapidly growing culture of non-lysogenic and non-resistant organisms.

Thus it is evident that the lysogenic property is intimately related to this irregular growth first described by Bordet and Ciuca (2). This is not only the case for *Bacillus coli* but it is a general rule, since similar observations have already been made by Kuttner for typhoid bacilli (3), by Wollstein for Shiga bacilli (4), and by myself for staphylococci (5).

It is evident also that irregular colonies are diseased colonies and that indentations must be considered as lesions. Our experiments show that when the diseased material is streaked on agar plates, the lesions are very numerous in the first streaks but gradually disappear in the following ones and that in the last streaks only perfectly regular, healthy, and non-lysogenic colonies are found.

It results also from the preceding observations that all the organisms composing a culture of *coli* O do not respond in the same way to the lytic agent. (1) Some are so sensitive that they are dissolved even by very dilute filtrate (spots of clarification, Fig. 1, Plate B). (2) Some are just resistant enough to survive and grow in the presence of a moderate quantity of lytic agent, but they are still weak organisms, and, as they are more or less affected by the lytic agent, they produce diseased irregular colonies. They are the lysogenic organisms of Bordet and Ciuca. When streaked on agar plates, they can recover and produce in the last streaks regular, large, flat colonies made up of sensitive and non-lysogenic organisms. (3) Only a few individuals are sufficiently resistant to survive the strong action of concentrated lytic agent. They are absolutely invulnerable to the lysis and produce regular, small, convex, and hyaline colonies of very resistant and non-lysogenic organisms (*coli* O R 2). (4) Among these very resistant bacilli only a very few are mucoid.

The three principal characteristics of the modified *coli* of Bordet and Ciuca, *i.e.* resistance, lysogenic properties, and mucoid growth, are thus shared among different organisms.

$$\textit{B. coli}\ \text{O} + \text{lytic agent} = \begin{cases} \text{Irregular lysogenic colonies} \\ \text{Regular non-lysogenic colonies} \begin{cases} \textit{Coli}\ \text{O R 1} = \text{Resistant, mucoid} \\ \textit{Coli}\ \text{O R 2} = \text{Resistant, non-mucoid} \end{cases} \end{cases}$$

Action of the Lytic Agent on coli S and coli R.

As described in a preceding paper (1), two different types of organisms, *coli* S and *coli* R, have been obtained by allowing *coli* O to age. Both types have lost the property of producing mucoid growth when submitted to the lytic agent.

Experiment 5.—6 hour broth cultures of *coli* O and *coli* R were added respectively to 0.2 cc. of lytic agent, and incubated at 37°C. When a loopful of each partially dissolved culture was planted on agar slants 36 hours later, a scanty and irregular culture resulted which, after several passages, grew more readily, indeed, but never became mucoid.

This result is confirmed and explained by the following experiment.

Experiment 6.—The technique used in Experiment 1 for *coli* O was applied to *coli* S and *coli* R. Again irregular lysogenic colonies were found in the plates containing weak lytic agent, and regular resistant colonies in the plates containing concentrated lytic agent. But in any case, in spite of a large number of experiments, no mucoid colonies have been found.

In other words, neither *coli* S nor *coli* R contains any mucoid organism, or, at least, any organism capable of becoming mucoid.

Another striking observation can be made in this experiment. On account of its greater resistance, *coli* R gives a greater number of round resistant colonies and only a very few irregular lysogenic colonies. Moreover, when streaked on agar plates, the irregular growth cannot be preserved any length of time; hence, at the end of three or four passages, the growth is almost completely regular. Thus, *coli* R not only is less affected by the lytic agent than the more sensitive strains, *coli* O and *coli* S, but also recovers more quickly.

As the ultimate result of the lytic agent on *coli* S and *coli* R we thus obtain resistant, non-lysogenic, non-mucoid organisms. These organisms we shall term *coli* S R and *coli* R R respectively. *Coli* S R is non-motile, and, when transplanted, grows very slowly, producing only a slight turbidity in broth and very small round hyaline colonies on agar plates. *Coli* R R, on the other hand, is very motile and grows quickly.

$$\underset{\text{(non-motile)}}{\textit{B. coli}\ \text{O} + \text{age}} = \begin{cases} \underset{\text{(non-motile)}}{\textit{Coli}\ \text{S}} + \text{lytic agent} = \text{Resistant } \underset{\text{(non-motile)}}{\textit{coli}\ \text{S R}} \\ \underset{\text{(motile)}}{\textit{Coli}\ \text{R}} + \text{lytic agent} = \text{Resistant } \underset{\text{(motile)}}{\textit{coli}\ \text{R R}} \end{cases}$$

Further Variations of the Modified coli.

An agar slant of modified *coli*, obtained by Bordet and Ciuca, was kept in the ice box for several months before being used in the following experiments.

Experiment 7.—The old modified *coli* was transplanted each day on agar slants. The culture remained mucoid and lysogenic.

Experiment 8.—A trace of the modified *coli* was streaked on an agar plate and after 12 hours incubation two types of colonies were found: the more numerous were mucoid, the others—only a few—were non-mucoid. Both types of colonies were round and non-lysogenic.

Experiment 9.—Both types were streaked respectively on agar plates, and since this first isolation two pure cultures have been obtained which breed true—a mucoid one, or *coli* M 1 (Fig. 4, B), and a non-mucoid one, or *coli* M 2.

$$\underset{\text{(non-motile)}}{B.\ coli\ \text{O}} + \text{lytic agent} = \text{Modified } B.\ coli + \text{age} = \begin{cases} \underset{\text{(motile, mucoid)}}{coli\ \text{M 1}} \\ \underset{\text{(motile, non-mucoid)}}{coli\ \text{M 2}} \end{cases}$$

As a matter of fact, it will be seen later that these strains cannot be identified with any of the types above described.

Characteristics and Evolution of coli M 2.

When streaked on agar plates, *coli* M 2 produces large, flat, pale blue, translucent, and non-fluorescent colonies. Different individuals of that type vary greatly in their motility, as observed by microscopic examination and by planting in semisolid medium. Submitted to the resistance test, they show an average resistance; *i.e.*, they do not possess the absolute invulnerability of *coli* R R or *coli* S R, but the path of clarification left by the drop on an agar slant of *coli* M 2 can be completely recovered by a resistant growth as early as 12 hours after seeding. The lysogenic test is negative. All of these characteristics are maintained indefinitely. When freshly isolated, *coli* M 2 is spontaneously agglutinable in plain broth. But this character is transient and disappears after a few passages in broth or on slanted agar.

The most interesting property of *coli* M 2 is its remarkable ability to give mucoid growth when again submitted to the lytic agent.

Experiment 10.—0.2 cc. of lytic agent was added to a broth culture of *coli* M 2 (6 hour, pH 8.0). A rather easy and almost complete dissolution occurred. After 24 hours incubation, a loopful of the partially dissolved culture was streaked on agar plates and gave the result shown in Fig. 4, A. In the first streak, in which the living bacilli and the lytic broth had been spread in abundance, the growth was scanty and irregular, but became less and less irregular in the following streaks, and was finally completely regular in the last streaks. This, of course, was to be expected from our former observations, but the remarkable fact was that a certain number of mucoid colonies was present in the first streak, very often covering the debris of an almost completely dissolved irregular colony of *coli* M 2 (Figs. 5 and 6).

Experiment 11.—Same experiment as the preceding one, but the sample of the dissolved culture was taken and streaked only after 3 days incubation. In this case almost all of the colonies were mucoid.

B. coli M 2 + lytic agent = *B. coli* M 1
(non-mucoid) (mucoid)

It may be concluded that different types of *coli* differ greatly in their ability to give a mucoid growth when submitted to the action of the lytic agent. Some, like *coli* S and *coli* R, lack this property entirely; others, like *coli* O, have it to a certain extent; and some, like *coli* M 2, have it to a very high degree.

Characteristics and Evolution of coli M 1.

Streaked on agar plates, *coli* M 1 gives very large colonies, extremely mucoid, opaque, and fluorescent. Like *coli* M 2, these organisms have a variable motility, an average resistance, and no lysogenic properties.

Experiment 12.—*Coli* M 1 was streaked every day on agar plates, and retained these characteristics even after a great number of passages. But occasionally a colony showed on a portion of its edge a kind of indentation where the growth was non-mucoid. This transformation of mucoid into non-mucoid growth has a very typical aspect which can be seen in Fig. 7.

Experiment 13.—A trace of the non-mucoid material of the indentation described in the foregoing experiment was streaked on agar plates and gave a pure culture of non-mucoid and non-motile organisms (*coli* M 1 a) which possesses absolutely all the characteristics of *coli* O, and which, therefore, can be considered as a reversion to the original type.

With the exception of this occasional reversion, *coli* M 1 is very stable on agar plates, since only mucoid colonies have been observed in the course of more than 50 platings. *Coli* M 1 is very stable also when kept growing in a synthetic medium, as the following experiment shows.

Experiment 14.—*Coli* M 1 was seeded in a tube of synthetic medium, composed as follows:

Water	1,000 cc.
Glycerol	30 cc.
Sodium chloride	5 gm.
Calcium chloride	0.1 gm.
Magnesium sulfate	0.2 gm.
Dipotassium phosphate	2.0 gm.
Ammonium lactate	12.0 gm.

pH 7.4

Transplantations were renewed daily. At the same time a loopful of each 24 hour culture was streaked on agar plates, in order to control the behavior of *coli* M 1 in the synthetic medium. Nothing but mucoid colonies was found in the plates in the course of more than fifteen passages of *coli* M 1 in synthetic medium.

Quite different was the result when *coli* M 1 was planted in plain broth.

Experiment 15.—Same experiment as the preceding one, but plain broth was substituted for synthetic medium. Since the first passage in plain broth a certain number of non-mucoid bacilli has appeared, as shown by the corresponding plate in which, among mucoid colonies, a certain number of non-mucoid colonies was found. At the end of ten passages in plain broth, almost all the bacilli were non-mucoid.

Plain broth realizes, then, a condition which does not exist in agar plates or in synthetic medium, and which induces the transformation of the mucoid *coli* M 1 into a non-mucoid form, or *coli* M 1 b.

This new non-mucoid form derived from *coli* M 1 in broth is not at all similar to the non-mucoid reversion, or *coli* M 1 a, which appears occasionally on agar plates. The first is extremely motile, while the latter is not. Nor is it similar to the motile non-mucoid modified *coli* described above under the denomination of *coli* M 2, because *coli* M 2, as we have seen, becomes very easily mucoid again when submitted to the lytic agent, while *coli* M 1 b never reverts to the mucoid form, even in the presence of lytic agent, as is shown in the following experiment.

Experiment 16.—5 drops of lytic agent were added respectively to 6 hour cultures of *coli* M 2 and *coli* M 1 b, and both mixtures were allowed to incubate at 37°C. for several days. A loopful of each mixture was streaked every day on agar plates in order to control the appearance of mucoid bacilli. While numerous mucoid bacilli have already appeared since the 1st day in the mixture of *coli* M 2 with lytic agent, not one could be detected in the mixture of *coli* M 1 b, even after 5 days of incubation.

$$\begin{array}{r}\textit{B. coli}\ \text{M 1}\\ \text{(mucoid,}\\ \text{motile)}\end{array}\left\{\begin{array}{l}\text{In synthetic medium} = \textit{Coli}\ \text{M 1}\\ \qquad\qquad\text{(mucoid, motile)}\\ \text{On agar plates} = \left\{\begin{array}{l}\quad\textit{Coli}\ \text{M 1}\\ \text{(mucoid, motile)}\\ \textit{Coli}\ \text{M 1 a} = \textit{Coli}\ \text{O}\\ \text{(non-mucoid, non-motile)}\end{array}\right.\\ \text{In plain broth} = \textit{Coli}\ \text{M 1 b}\\ \qquad\text{(non-mucoid, motile)}\end{array}\right.$$

A striking characteristic of *coli* M 1 b is that of giving an extraordinary granular growth when freshly isolated and transplanted in broth. Soon after transplantation, heavy clumps appear, which sediment at the bottom of the tube, leaving a clear supernatant broth.

This property led us to make a rather unexpected parallel with the recent observations of De Kruif on the bacillus of rabbit septicemia (6). This author observed the dissociation of the bacillus into two types: one is extremely virulent, produces opaque and very fluorescent colonies on agar plates, and grows diffusely in broth (Type D); the other type is avirulent, produces translucent and weakly fluorescent colonies, and gives granular growth in broth (Type G).

The similarity with our last findings is evident. Like Type D, *coli* M 1 gives opaque, very fluorescent colonies, and grows diffusely in broth; on the other hand, *coli* M 1 b, like Type G, gives translucent and weakly fluorescent colonies and produces granular growth in broth. According to recent experiments of De Kruif, Type D is able to change into Type G when kept growing in broth; *coli* M 1 b also appears in broth cultures of *coli* M 1, as we have seen. Type G never reverts to Type D; similarly no reversion from *coli* M 1 b to *coli* M 1 has thus far been observed, even in the presence of lytic agent. Because of this parallelism, it seems that the facts pointed out by

De Kruif are not a special feature of the bacillus of rabbit septicemia, but have a more general bearing.

Besides, our attention has been called repeatedly to similar facts reported by Arkwright (7), who found that *Bacillus dysenteriæ*, *Bacillus typhosus*, *Bacillus paratyphosus*, and *Bacillus enteritides*, could be made to yield two forms, the rough ("R") form, which makes stable emulsions, and the smooth ("S") form, which is spontaneously agglutinable. This distinction is similar to that, for instance, in the case of *coli* M 1 and *coli* M 1 b, and also to that in the case of *coli* M 1 and *coli* M 2.

General Properties of the Different Types.

In the course of our studies on the transmissible autolysis of *Bacillus coli*, we have thus isolated ten different types of organisms, all derived from the original *Bacillus coli* of Bordet and Ciuca. They are perfectly distinguished from each other by striking characteristics, such as size, shape, opacity and fluorescence of colonies, mucoid or non-mucoid growth, ability to yield mucoid growth in the presence of lytic agent, motility, resistance to desiccation and to lytic agent, etc. (Table I). The specific properties of the colon bacillus are preserved; namely, the fermentation of carbohydrates, with the exception of saccharose, and the production of indole. The following experiment deals with the agglutination reaction.

TABLE I.

Type.	Resistance.	Motility.	Mucoid growth.	Ability to yield mucoid growth.	Fluorescence.	Seroagglutination.
O	+	0	0	+	++++	0
O R 1	++++	0	++++		0	
O R 2	++++	0	0	0	0	
S	+	0	0	0	++	+++
S R	++++	0	0	0	0	+++
R	++	++++	0	0	++	++++
R R	++++	++++	0	0	0	++++
M 1	+++	+++	++++		++++	++++
M 1 a	+	0	0	+	++++	0
M 1 b	++	++++	0	0	++	++++
M 2	+++	+++	0	++++	0	++++

Experiment 17.—Rabbits were immunized respectively with *coli* O, *coli* S, and *coli* R. The agglutinating power of the three antisera obtained was tested on nine of our strains by the following technique advised by Bordet and Ciuca. Increasing dilutions of the three antisera were made with plain broth and 1 cc. of each dilution of each serum was seeded respectively with one of the nine strains and incubated at 37°C. When the bacilli grew in clumps, the result was considered as positive. When the growth was diffuse the result was considered negative. The results are shown in Table II.

TABLE II.

Antiserum.	Titer of antiserum.	Strain.								
		O	S	S R	R	R R	M 1	M 1 a	M 1 b	M 2
O	1:10	−	++	+	+++	+++	+++	−	+++	+++
	1:100	−	+	−	+++	+++	++	−	++	−
	1:500	−	−	−	++	++	+	−	+	−
	1:1,000	−	−	−	−	−	−	−	−	−
	1:5,000	−	−	−	−	−	−	−	−	−
S	1:10	−	+++	+++	++	++	+++	−	++	++
	1:100	−	+++	+++	++	++	+++	−	++	++
	1:500	−	++	++	+	++	+++	−	++	++
	1:1,000	−	+	−	+	+	+++	−	+	++
	1:5,000	−	−	−	−	−	+++	−	+	++
R	1:10	+	+++	+++	+++	+++	+++	+	+++	+++
	1:100	+	+++	+++	+++	+++	+++	+	+++	++
	1:500	−	+++	+++	+++	+++	+++	−	+++	++
	1:1,000	−	+++	++	+++	+++	+++	−	+++	++
	1:5,000	−	++	−	+++	+++	+++	−	++	++

Two interesting facts appeared in this experiment. (1) While all the other strains grew in clumps in the presence of any of the three antisera, only the original *coli* (*coli* O) and the reversion to the original type (*coli* M 1 a) were not agglutinable, even by their corresponding antiserum, *i.e.* the serum obtained from a rabbit immunized with *coli* O, which, however, agglutinated the other types. It is very interesting that Type M 1 a, which we already considered as a reversion on account of its similarity with the original *Bacillus coli*, shows the same lack of agglutinability as *coli* O. (2) The titer of the anti-*coli* S serum and of anti-*coli* R serum was greater than 1:5,000, while, on the other hand, the power of anti-O serum was much lower and hardly reached a titer of 1:500. *Coli* O is not only non-agglutinable but possesses also weak antigenic power.

DISCUSSION.

What is the nature of the different variations above described?

The notion of contamination can be disregarded with certainty, not only in consideration of the care constantly taken but because it has been possible to reproduce the experiments at any time, always with the same expected result, and because a certain number of our types have been isolated simultaneously in Brussels by Bordet and Ciuca from the same original strain of *Bacillus coli.*

The range of the variation never goes beyond the limit of the species: all of the strains still possess the specific properties of *Bacillus coli.*

All of the types, once isolated and regularly transplanted, keep their individuality even after several months. They are thus stable. Nevertheless, they are not all uniformly stable, and certain types, like *coli* M 1, for instance, still readily undergo changes under certain definite influences.

It is out of the question also to say that the eleven types coexisted in the original strain, because in order to admit such a coexistence it would be necessary to imagine that at least eleven different organisms, each of them representing one of our variations, came together through the three successive isolations performed before the present studies were begun. We have thus been forced to accept the conclusion that the different types observed are the result of changes occurring in the original *Bacillus coli* in the course of these studies. The new types always appear under certain definite conditions of the environment, but it is still impossible to determine whether the external influence is the direct cause of the variation or only an occasional factor which makes apparent, by mere selection, a modified germ already present but in too small numbers to be detected, and produced independently of the environment itself.

It is a common observation that transmissible lysis of a bacterium, induced under certain influences such as stool filtrate, peritoneal exudate, tissue extracts, and vaccine, happens in a rather haphazard and irregular fashion and seems a question of luck. This fact is easily explained by the hypothesis of a bacteriophage virus. But if the phenomenon is not due to a virus, but as suggested by Bordet and Ciuca, to some autolytic vitiation of the bacteria, the irregularity with which the phenomenon is promoted must find its cause in the bacteria themselves. It is possible that a given bacterium is not always capable of starting the lysis. Various forms of *Bacillus coli* appear to differ in their properties, for example in their behavior toward the lytic agent. Certain forms become mucoid in the presence of the lytic agent. Similarly, it may be possible for only a certain form to undergo autolysis. In order to start the dissolution it is necessary to employ the proper forms of bacteria.

SUMMARY.

1. When the few individuals still alive in a dissolved culture of *Bacillus coli* are transplanted on slanted agar, a culture results which possesses new characteristics. First observed by Bordet and Ciuca, this culture received the temporary name of modified *coli*.

In the study described above, we found that this modified *coli* is very heterogeneous and that its three principal characteristics, resistance to lysis, lysogenic properties, and mucoid growth, are shared among different types of organisms that can be isolated when the normal original *coli* (*coli* O) is plated together with increasing quantities of the lytic agent: (*a*) a certain number of bacilli are just resistant enough to survive and grow in the presence of a moderate quantity of lytic agent, but they are still more or less sensitive and produce diseased, irregular, and lysogenic colonies; (*b*) a few of the organisms are able to resist concentrated lytic agent; they are entirely resistant and give round, healthy, and non-lysogenic colonies (*coli* O R 2); and (*c*) among these resistant bacilli only a very few are mucoid (*coli* O R 1). All these types are not motile and not fluorescent.

2. The original *coli*, when allowed to age, can be dissociated, as we have shown in a preceding paper (1), into two types of organisms, the non-motile *coli* S and the very motile *coli* R. Submitted to lysis, *coli* S gives a very small number, *coli* R a much greater number of resistant organisms (*coli* S R and *coli* R R), but both types never yield any mucoid growth.

3. An old culture of the modified *coli* obtained by Bordet and Ciuca, when streaked on agar plate, gives two types of colonies: a mucoid and fluorescent type (*coli* M 1) and a non-mucoid and translucent type (*coli* M 2). Both types are motile.

Coli M 2, once isolated, keeps its individuality even after several passages in artificial media, but if again submitted to the lytic agent, a great number of mucoid bacilli are found among the organisms which are still alive.

Consequently, different types of *Bacillus coli* differ greatly in their ability to give a mucoid growth when submitted to the lytic agent. Some, like *coli* S and *coli* R, do not possess this property at all. Others, like *coli* O, possess it to a certain extent, and some, like *coli* M 2, have it to a very high degree.

4. The mucoid and motile *Bacillus coli* M 1, when streaked every day on agar plates, remains indefinitely mucoid and motile, but occasionally a mucoid colony shows an indentation made up of non-mucoid growth, which, transplanted, gives a pure culture of non-mucoid and non-motile organisms, *coli* M 1 a. This new type possesses all the characteristics of the original strain of *Bacillus coli*, and therefore must be considered as a reversion.

5. The mucoid and motile *Bacillus coli* M 1, kept growing in synthetic medium, remains perfectly stable; on the other hand, when it is transplanted in broth, *Bacillus coli* M 1 turns very quickly into a non-mucoid but still very motile organism, or *Bacillus coli* M 1 b. This last type, which produces translucent colonies on agar and grows granular in broth, never reverts to the mucoid form, even in the presence of lytic agent.

6. A single strain of *Bacillus coli* has thus been made to yield eleven different forms, all distinguished by striking characteristics, but still possessing the specific properties of *Bacillus coli.*

Nine of these forms have been submitted to antisera prepared with three different types (*Bacillus coli* O, *Bacillus coli* S, and *Bacillus coli* R). While seven out of these nine strains were agglutinated by any of the three antisera, only the original *Bacillus coli* (*Bacillus coli* O) and the reversion to the original type (*Bacillus coli* M 1 a) were not agglutinable, even by their corresponding antiserum; *i.e.*, the serum obtained from a rabbit immunized with *Bacillus coli* O, which, however, agglutinated the other types.

BIBLIOGRAPHY.

1. Gratia, A., *J. Exp. Med.*, 1921, xxxiv, 115.
2. Bordet, J., and Ciuca, M., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1293, 1296.
3. Kuttner, A., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 158.
4. Wollstein, M., *J. Exp. Med.*, 1921, xxxiv, 467.
5. Gratia, A., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 217.
6. De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.
7. Arkwright, J. A., *J. Path. and Bact.*, 1921, xxiv, 36.

EXPLANATION OF PLATES.

PLATE 7.

FIG. 1. Experiment 1. *Coli* O plated with increasing quantities of lytic agent (O, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}). Plate A. Control; normal culture of *coli* O. Plates B, C, and D. *Coli* O + moderate quantities of lytic agent; note the great number of irregular colonies. Plates E and F. *Coli* O + concentrated lytic agent; note the presence of only small regular colonies. No mucoid colonies are found in this experiment.

PLATE 8.

FIG. 2. When Experiment 1 is repeated several times, a mucoid colony can occasionally be found. Here is a plate of *coli* O mixed with concentrated lytic agent. Besides the small regular colonies, two large mucoid colonies are visible.

FIG. 3. Plate A. A small round colony of Plate F, Fig. 1, was planted and gave a pure culture of *coli* O R 2. Plate B. The lower mucoid colony seen in Fig. 2 was transplanted and gave a mucoid culture of *coli* O R 1; note the translucent growth studded with opaque spots.

FIG. 4. Plate A. Experiment 10. A loopful of a partially dissolved culture of *coli* M 2 was streaked on an agar plate. In the first streak the growth is very irregular and contains a certain number of mucoid colonies. In the second, the colonies have already become less irregular, and in the third they are normal. Plate B. Mucoid growth of *coli* M 1.

FIG. 5. Magnification of a portion of the first streak of Plate A, Fig. 4. Showing mucoid colonies among the very irregular colonies of *coli* M 2.

FIG. 6. The same. A mucoid colony recovering the debris of a colony of *coli* M 2.

FIG. 7. Magnification of the edge of a colony of *coli* M 1 where an indentation of non-mucoid growth (light) issues from the mucoid growth (dark).

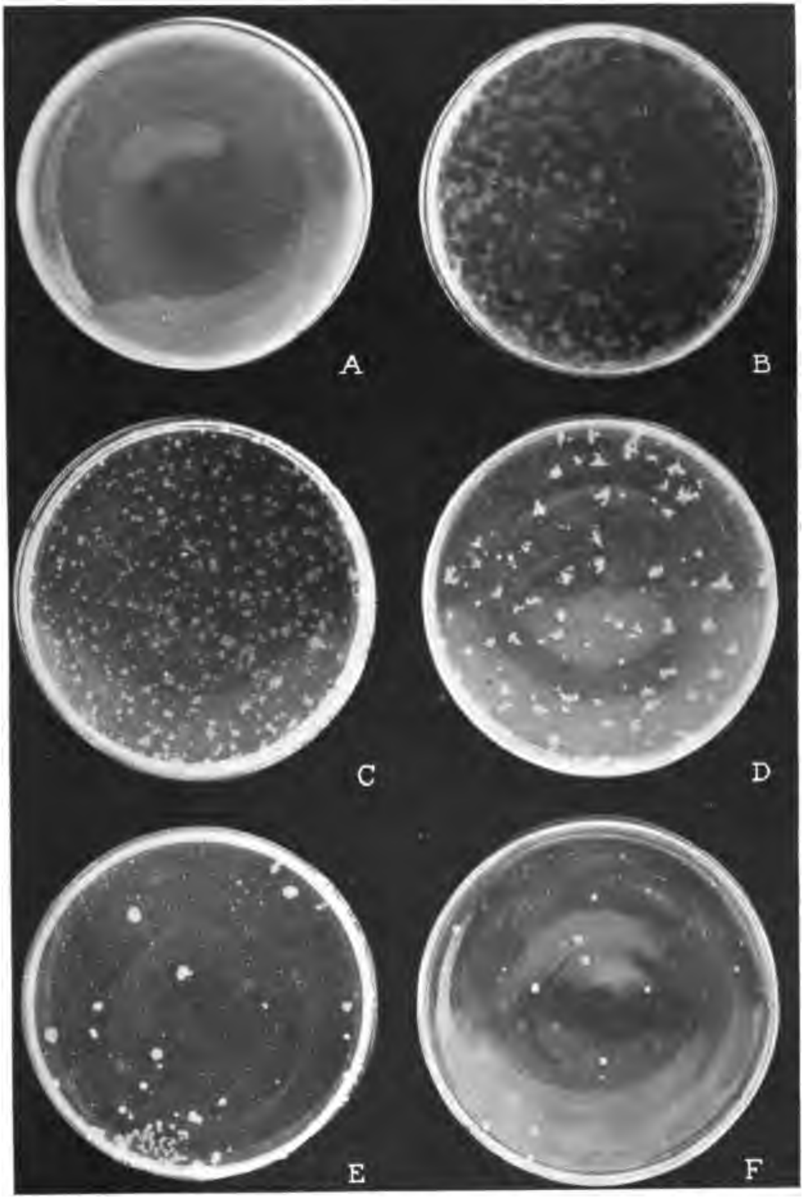


FIG. 1.

(Gratia: Twort-d'Hérelle phenomenon. II.)

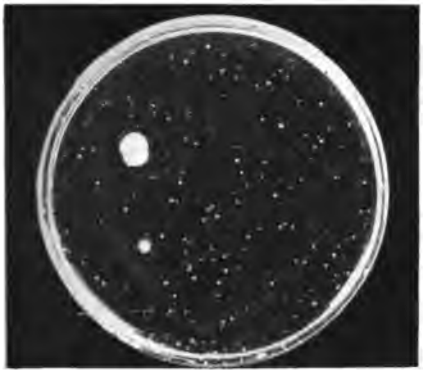


Fig. 2.

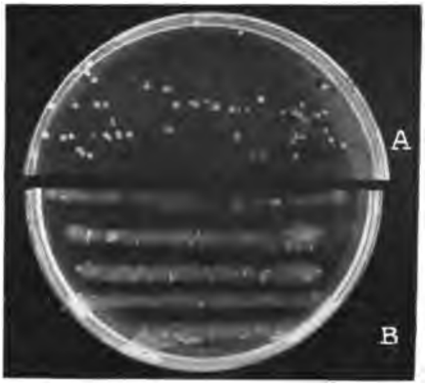


Fig. 3.

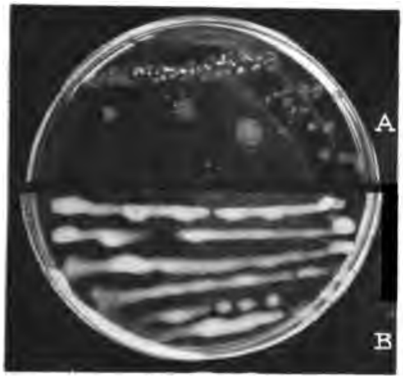


Fig. 4.

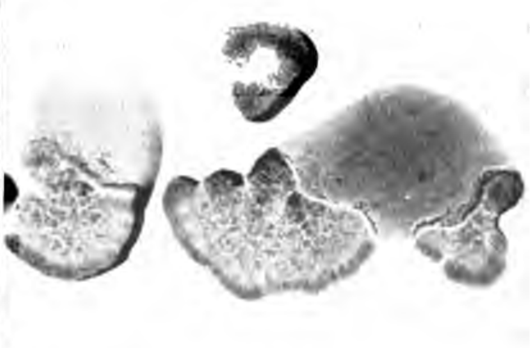


Fig. 5.

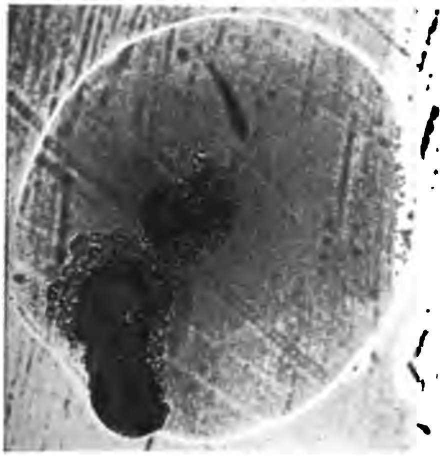


Fig. 6.

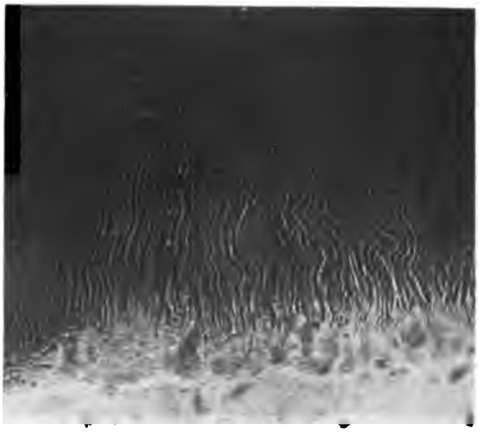


Fig. 7.

(Gratia: Twort-d'Hérelle phenomenon. II.)

THE CULTIVATION AND BIOLOGICAL CHARACTERISTICS OF SPIROCHÆTA OBERMEIERI (RECURRENTIS).

BY I. J. KLIGLER, PH.D., AND O. H. ROBERTSON, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 1, 1921.)

The spirochetes of relapsing fever were first cultivated by Noguchi[1] in 1912. He employed a culture medium consisting of ascitic fluid to which a fragment of fresh sterile rabbit kidney had been added. Noguchi placed considerable stress on the character of the ascitic fluid used, selecting only such as gave fibrin formation throughout the medium after inoculation. The culture fluid was inoculated with infected rat blood, and covered with a layer of paraffin oil. By this method he succeeded in growing these organisms through a number of generations. The maximum growth occurred between the 4th and 6th days, at which period subcultures were made. This technique was successfully applied by Plotz[2] in cultivating these spirochetes from the blood of patients. Subsequent to Noguchi's report, Hata[3] cultivated *Spirochæta obermeieri* in horse serum diluted with saline solution and coagulated at 70°C. He found that in the presence of kidney the organisms lived only a short time, but that on substituting a fragment of blood clot for kidney, they survived and remained infective for a long period. Recently Ungermann[4] has reported successful results with inactivated rabbit serum without kidney.

The principle underlying the cultivation of *Spirochæta* was discovered by Noguchi. However, there are certain details in the preparation of the medium and the conditions controlling the growth of these organisms which had not been fully studied.

In our attempts to cultivate the spirochetes of relapsing fever by the different methods outlined above, we found the Noguchi method the most satisfactory, but in our hands the results were inconstant. Occasionally a good initial growth was obtained, but it was not always possible to carry the culture to a second generation. Frequently in the same medium, either no growth occurred in spite of a rich inoculum, or an abundant growth was suddenly terminated by rapid degeneration at the end of the 3rd or 4th day. It was evident that we were dealing with certain unknown variables in the culture media which in one instance made it suitable and in another unsuitable for the growth of the spirochetes. Furthermore, it was evident that even in a favorable medium changes were rapidly taking place which rendered it unfavorable. Therefore, it seemed desirable to analyze more fully the factors in the growth requirements of these organisms. *Spirochæta obermeieri* was the type used in these experiments.

---

[1] Noguchi, H., *J. Exp. Med.*, 1912, xvi, 199.

[2] Plotz, H., *J. Exp. Med.*, 1917, xxvi, 37.

[3] Hata, S., *Centr. Bakt.*, *1te Abt.*, *Orig.*, 1914, lxxii, 107.

[4] Ungermann, E., *Arb. k. Gsndhtsamte*, 1919, li, 114.

Culture Media.

Constituents of the Media.—Our findings, with regard to the basic constituents of the most favorable culture media, agreed in the main with those of former workers, and may be summarized briefly as follows:

Animal fluids are necessary for the growth of *Spirochæta obermeieri*. Ascitic fluid, horse and rabbit serum serve this purpose equally well. Inactivation of the serum was found to be unnecessary, and coagulation undesirable. Dilution of the serum with one to two parts of normal salt solution does not impair its nutrient properties. Growth is more rapid in undiluted serum, but the viability of the culture is greater in diluted serum. The addition of kidney is not necessary.

The physical condition of the medium seems to exert an important influence. The growth activity of the culture is markedly increased by the presence of a small amount of agar or fibrin, as suggested by Noguchi, who found them favorable for *Leptospira icteroides*. Under these conditions, growth is more abundant and the spirochetes are found chiefly enmeshed in the agar or fibrin.

Reaction of the Media.—Recent investigations have shown that the concentration of hydrogen ions in the medium is an important limiting factor in affecting the growth and viability of microorganisms. Consequently a series of experiments was made to determine the effect of pH on the growth of the spirochetes. Media made up of ascitic fluid, horse and rabbit serum respectively were adjusted to various reactions, inoculated with the same amounts of infected rat blood, covered with oil, and incubated. After 3 to 5 days the cultures were examined under the dark-field microscope and the reaction of the media was again tested. The following protocol is typical.

Horse and rabbit sera, to which 0.1 per cent agar had been added, were adjusted to different reactions by the addition of N NaOH or N HCl. 3 cc. of medium were put into a tube. All the tubes were inoculated with the same amount of infected rat blood and incubated at 30°C. for 3 days. The data are presented in Table I.

TABLE I.

Effect of the Reaction on Growth.

Tube No.	Rabbit serum.			Horse serum.		
	Initial pH.	Final pH.	Results.	Initial pH.	Final pH.	Results.
1	7.9	8.2+	0 Spirochetes dead.	7.9	8.2+	0 Spirochetes dead.
2	7.6	8.1	± 1 to 10 fields; degenerated.	7.6	8.1	± 1 to 10 fields; degenerated.
3	7.3	7.6	++ 1 or more to a field; active; in chains.	7.2	7.5	+++ 2 to 3 in a field; active; in chains.
4	6.9	7.1	+ 1 to 3 fields; active.	6.9	7.2	+++ 2 to 3 in a field; active.
5				6.4	6.6	0 None found.

These tests established two important facts: (1) that the reaction of the medium has a decided influence on the growth of the spirochete; and (2) that during the incubation, the reaction of the culture fluids changes progressively toward alkalinity irrespective of the initial reaction.

In media having a reaction below pH 7.0 or above pH 8.0 there was apparently no growth, while the maximum rate of multiplication occurred between pH 7.2 and 7.4. Even in an actively growing culture rapid degeneration set in as soon as the reaction approached the limiting pH. This has been verified by repeated tests of the reaction of cultures which had suddenly died.

The progressive increase in the alkalinity of the media (Table I) is a factor of considerable importance in the cultivation of these organisms. This change occurred irrespective of the initial reaction of the medium or of the growth of the spirochetes, and was characteristic of all media made up largely of animal fluids. It was found that ascitic fluid, horse, sheep, and rabbit sera become progressively more alkaline after withdrawal from the body and exposure to the air. Felton, Hussey, and Bayne-Jones[6] observed that spinal fluid changed from a pH 7.7 or 7.8 to 8.6 within a few hours. Ascitic fluid is also prone to marked rapid change, while animal sera change more slowly. The following experiment is illustrative.

Horse and rabbit sera were diluted with saline solution in the proportion of 1:3, the reactions were adjusted, and the fluid was distributed in 3 cc. amounts into a series of tubes. Tubes of ascitic fluid were also made. Another similar set of tubes was set up with serum and ascitic fluid which had been heated for 10 minutes in a water bath at 100°C. The results, after incubation for 2½ days, are given in Table II.

TABLE II.

Reaction Changes in Animal Fluids at Room Temperature.

Incubation time 2½ days.

Fluid.	Unheated.		Heated.	
	Initial pH.	Final pH.	Initial pH.	Final pH.
Horse serum (1 : 3)	7.0	7.8	7.0	7.8
Rabbit " (1 : 3)	7.2	7.7	7.2	7.8
Ascitic fluid	7.2	8.2+	7.2	8.2+
" " (1 : 1)	7.3	8.2+	7.3	8.2+

The instability of the reaction is most likely due to a progressive loss of CO_2. This is indicated by the facts that the change is always toward alkalinity, that agitation hastens the change, and, finally, that heating to destroy enzymes does not prevent or even retard the reversion. The loss of CO_2 would readily account for the increase in alkalinity. The relationship of CO_2 concentration in the blood and blood reaction has been fully discussed by L. J. Henderson.[6]

---

[6] Felton, L. D., Hussey, R. G., and Bayne-Jones, S., *Arch. Int. Med.*, 1917, xix, 1085.

Stabilization of the Media.—It was evident that the next step was to find some means of stabilizing the culture fluids. Several methods suggested themselves, each of which was tested in turn.

1. The first procedure was to acidify the medium, agitate vigorously, and then adjust to the desired reaction. This led to the precipitation of the serum proteins which were not completely redissolved. The turbid fluid was not satisfactory for culture purposes.

2. We then determined the extent to which kidney tissue, as used by Noguchi, tends to regulate the reaction. It is well known that kidney tissue autolyzes with the liberation of acid, but the degree and rate of autolysis and diffusion of the acid were unknown. It was found that the kidney tissue gradually neutralizes the alkalinity of the medium but that diffusion is slow and the effect is not uniform on account of the number of variables that enter. As a result of the slow diffusion of the acid, the portion of the medium above the kidney may be decidedly acid, the upper portion still alkaline, while in between is a zone of graded reactions. The rate and degree of acidification depend roughly on the relation of the size of the kidney tissue to the amount of fluid used, as well as on the character and initial reaction of the fluid. With a piece of kidney of appropriate size and a suitable amount of animal fluid of a given reaction, the final reaction of the medium may be brought to about the desired level. For example, a fragment of kidney tissue, the size of a pea, added to 6 cc. of ascitic fluid of a reaction of pH 8.4 brought the reaction down to a pH of 7.2 in 2 days; a larger piece of kidney with the same amount of this ascitic fluid made the medium decidedly acid. But one could not predict in any given case what the final reaction would be. Noguchi's results with this method were presumably due to a proper proportion between ascitic fluid, reaction, and kidney tissue, and the fact that he made transplants before the reaction of the media had changed beyond the limits of growth.

3. Mineral oil as originally used by Noguchi was also found to retard the change in reaction. The higher the column of oil the

---

[6] Henderson, L. J., *J. Biol. Chem.*, 1909–10, vii, 29.

less rapid the change. However, as shown in Table IV, the oil itself is not sufficient to prevent rapid alkalization of the medium to a point inhibitive to the growth of the spirochete.

4. Since the increase in alkalinity is presumably due to diffusion of CO_2, it seemed likely that a simple solution to the problem would be growing the organism in an atmosphere containing an appropriate amount of CO_2. The stabilizing effect of CO_2 is illustrated by the following experiment.

TABLE III.

Effect of CO_2 on the Reaction.

Changes of Media; Initial Reaction pH 7.3.

Incubation time 3 days. All tubes kept in air-tight jar under the same conditions.

Media.	Dextrose broth cultures of *B. coli.*	3 vol. per cent CO_2.	5 vol. per cent CO_2.
Ascitic fluid	7.7	7.4	7.3
	7.7	7.5	7.3
Serum (1 : 2)	7.3	7.3	7.3
	7.3	7.3	7.3
Ascitic fluid + broth	7.5	7.3	7.3
" " + phosphate	7.3	7.3	7.3
Serum (1 : 2) + broth	7.3	7.3	7.3
Control without *coli* or CO_2.			
Ascitic fluid	8.0+		
" " + 1 per cent peptone	7.6		
" " + 1 " " egg albumin	7.8		

Test-tubes containing various kinds of culture fluids were placed in glass-topped jars. Into some of the jars were put dextrose broth cultures of *B. coli*, while to others different volumes per cent of CO_2 gas were added. The results are shown in Table III.

Similar experiments to test the ability of cultures to grow under these conditions gave satisfactory results. A simple procedure was to adjust the reaction of the ascitic fluid to pH 7.2, inoculate with infected blood, cover with about 1.0 cm. of oil, and put in jars together with two flasks of dextrose broth inoculated with *B. coli.* Good growth was obtained in 4 to 5 days. The only objection to this method is that it requires resetting of jars every time a culture is examined.

5. Finally, the reaction of the medium was stabilized by the addition of buffer substances. Peptone, egg albumin, and phosphates were used. All had the desired effect, to a greater or lesser extent (Table IV), but phosphates in amounts greater than 0.2 per cent were somewhat toxic to the spirochete, while egg albumin was too weak a buffer. Peptone apparently met all the requirements.

TABLE IV.

Effect of Buffer and of Various Amounts of Oil on the Reaction.

Effect of buffer. Initial pH 7.2; time 5 days.		Effect of oil. Initial pH 7.4; time 2 days.	
Media.	Final pH.	Media.	Final pH.
Ascitic fluid....................	8.2+	Ascitic fluid.	9.0
" " + 2 cm. of oil.....	8.0	" " + 1 cm. of oil.	7.9
" " + 1 per cent peptone.......................	7.8	" " + 2 " " "	7.9
Ascitic fluid + 1 per cent egg albumin....................	8.2	" " + 3 " " "	7.8
Ascitic fluid + 0.1 per cent phosphate.......................	8.0	" " + 4 " " "	7.7

Limiting Hydrogen Ion Concentration.

In the early experiments it was indicated that the reaction limits for the growth of *Spirochæta obermeieri* were pH 7.0 and 8.0 with an optimum at pH 7.2 to 7.4. On account of the shifting reaction during incubation, it was not possible to determine these limits accurately. Repeated tests with buffered media have given practically the same results. No growth was obtained in media having a pH value of 6.8 on the one hand, or 8.4 on the other. Growth takes place in the zone between pH 7.0 and 8.2, but is always most active at pH 7.3.

The following is a typical experiment.

Ascitic fluid containing 2 per cent peptone broth was divided into various lots, each adjusted to a different pH, and the media were distributed into tubes. Each tube was inoculated with the same amount of infected blood. After incubating 4 days at 30°C. the observations in Table V were made.

TABLE V.

Limiting Reactions of Growth of Spirochæta obermeieri.

Medium: Ascitic fluid + 2 per cent peptone broth. Incubation time 4 days.

Initial pH.	Final pH.	Results.
6.8	6.9	± An occasional live organism; many dead.
7.0	7.1	++ Active; chains of 2; 1 in 5 or 6 fields.
7.0	7.3	+++ Many active; chains of 2 and 3 elements; 1 to 2 per field.
7.4	7.5	++ Many active; 1 in 2 or 3 fields.
7.6	7.7	++ Active; chains of 2; 1 in 5 or 6 fields.
7.8	7.9	+ Fewer active; some degenerated.
8.0	8.1	+ About the same as 7.8.
8.2	8.3	± Many degenerated; occasional active spirochete.

Oxygen Requirements.

Some uncertainty exists regarding the oxygen requirements of these spirochetes. Noguchi[7] recognizes their essential need of oxygen and calls them aerotropic anaerobes. Ungermann,[4] on the other hand, considers them strict anaerobes. The idea that a layer of paraffin oil serves as an oxygen barrier is entirely wrong. Our early observations indicated that they were strictly aerobic, and the following experiments show this to be the case.

---

[7] Noguchi, H., *The Harvey Lectures*, 1915-16, xi, 236.

(*a*) *Oxygen Tension.*—Cultures of *Spirochæta obermeieri* were prepared and placed in a series of Noguchi jars. These jars were then exhausted and refilled with definite proportions of O and N gas, measured by means of a vacuum gauge, to give varying oxygen tensions. The jars were left at room temperature (27—30°C.) for 4 days, after which the cultures were examined.

A typical protocol follows.

Oxygen, *vol. per cent*	40	20	18	14	9	5
Growth.	0 A few surviving organisms; degeneration.	++ Good growth. 1 to 2 to a field; few degenerated.	+ 1 to 5 or 6 fields; many degenerated.	0 Few surviving; marked degeneration.	0 Few surviving; marked degeneration.	0 Few living.

The best growth was obtained under normal oxygen tension. A decrease to 0.9 per cent atmospheric tension retards growth, while a greater decrease or increase in the amount of oxygen apparently prevents growth entirely.

(*b*) *Oil Layer.*—Since a layer of oil had been found of value in stabilizing the reaction of the medium, it was important to determine the maximum column that might be used without preventing adequate aeration. It was found, contrary to Ungermann's assumption, that oil does not exclude air, but apparently retards the diffusion of gases. In a series of cultures made with increasing columns of oil, most abundant growth was obtained in those tubes having a layer 0.8 to 1.5 cm. high. Presumably a column of oil greater than that is sufficient to prevent the normal ingress of air.

(*c*) *Column of Media.*—The height of the column of medium was found to be of comparatively little importance. This was to be expected since growth is always most abundant in the upper, well aerated portion of the column. However, it was found that, other conditions being the same, growth was better when the column of the medium did not exceed 4 to 5 cm. in height.

Method of Cultivation.

The method finally adopted for the cultivation of *Spirochæta obermeieri* is as follows:

TABLE VI.

Detailed History of Some Cultures and Transplants.

1. Horse Serum without Buffer.

Medium.*	Date inoculated.	Interval before examination.					
		4 days.	7 days.	10 days.	14 days.	21 days.	39 days.
Horse serum + 0.1 per cent agar.	Sept. 22	+ Few active.	++ Active forms free and in agar; many dead.	+++ Large numbers in agar; few free swimming; many dead.	++ Many live; more dead.	+ Few active; many dead in agar.	0 Culture dead.

2. Horse Serum with Buffer (Broth).

Medium.*	Date inoculated.	Interval before examination.						
		4 days.	7 days.	10 days.	14 days.	21 days.	39 days.	51 days.
Horse serum + 0.5 per cent broth† + 0.1 per cent agar.	Sept. 22	+++ Numerous live organisms in agar; some free swimming.	+++ Many active in agar and free; some dead.	+++ Mostly in agar; many dead.	++ Many live spirochetes, but increasing number dead.	+ Few active; more dead.	± Some active forms left.	0 Culture dead.

3. Horse Serum with Buffer (Peptone).

Medium.*	Date inoculated.	Interval before examination.			
		5 days.	10 days.	17 days.	23 days.
Horse serum + 1 per cent peptone + fibrin.‡	Sept. 24	++ Many active; few dead.	++ Chains; active; chains in 1 colony.	+ Few active.	0 Contaminated.

4. Horse Serum Diluted, with Buffer (Broth).

Medium.*	Date inoculated.	Interval before examination.					
		5 days.	10 days.	23 days.	37 days.	49 days.	61 days.
Horse serum + normal salt solution 1:1 + 0.5 per cent broth + fibrin.	Sept. 24	++ Many active; degenerated forms but none dead.	++ Active; chains; few degenerated.	+ Active; chains; many dead.	+ Active; colony.	+ Masses of active organisms in fibrin.	0 Contaminated.

5. Horse Serum Diluted, with Buffer (Egg Albumin).

Medium.*	Date inoculated.	Interval before examination.						
		5 days.	10 days.	17 days.	23 days.	37 days.	49 days.	61 days.
Horse serum + normal salt solution 1:1 + 1 per cent egg albumin + fibrin.	Sept. 24	++ Active; no dead forms seen.	+++ Chains of 2 and 4; masses of organisms.	+ Only occasionally free; mostly in agar; many dead.	+ Some active; many dead.	+ Few active; many dead.	+++ Free swimming; many active in fibrin. (Culture used for transfer and reaction test.)	
Horse serum + normal salt solution 1:2 + 1 per cent egg albumin + fibrin.	Sept. 24	+ Few live, active; none dead.	+ Few active; chains.	++ Many active colonies in fibrin.	+ Some active; many dead.	+ Few active; many dead.	+ Few active; contaminated.	0

---

* Reaction adjusted to pH 7.2.

† The broth added contained 10 per cent peptone, so that the amount of peptone in the culture fluid was 0.5 to 1.0 per cent according to the amount of broth added.

‡ 1 drop of fresh rabbit blood. All tubes covered with 1 cm. of oil and grown at room temperature after the first 3 days.

TABLE VI—*Concluded.*

6. *Ascitic Fluid with Buffer (Egg Albumin).*

Medium.*	Date inoculated.	Interval before examination.						
		5 days.	10 days.	17 days.	23 days.	37 days.	49 days.	61 days.
Ascitic fluid + 1 per cent egg albumin + fibrin.	Sept. 24	++ Active single forms and chains of 2; some dead and degenerated.	+ Active; chains of 2 and 3.	++ Many chains; active.	+++ Many active chains; masses in fibrin.	+ Few active; many dead.	+ Some active; mostly dead.	0 Contaminated.

Transplants (Cultures Carried for 4 Months).

Serum or ascitic fluid + broth.

Generation. 1st 2nd 3rd 4th 5th 6th 7th
Date inoculated. Sept. 24 → Oct. 11 → Oct. 23 → Oct. 31 → Nov. 12 → Dec. 13 → Jan. 6 → Jan. 26 discontinued.
Interval. 17 days 12 days 8 days 12 days 31 days 24 days 20 days.

The basic media consist of horse serum or rabbit serum diluted with one or two parts of saline solution, or of ascitic fluid undiluted. To the fluid used 1.0 per cent peptone broth is added (1.0 cc. of a 10 per cent peptone broth to 10 cc. of fluid), and the reaction adjusted to pH 7.2. The media are distributed in 3 to 4 cc. amounts into tubes approximately 1.0 cm. in diameter. The tubes are then inoculated with 1 drop of infected blood, or, if subcultures are being made, with 0.1 cc. of culture, and covered with a layer of oil about 1.5 cm. high. In initial cultures the rat blood furnishes the fibrin; in subcultures the fibrin is supplied by a drop of fresh rabbit blood added prior to the addition of the oil. By rotating the tube gently to obtain uniform distribution of the blood, a delicate fibrin basket forms which remains suspended from the oil. In place of fibrin 0.05 to 0.1 per cent agar may be used. The tubes are then incubated at a temperature of 28–30°C.[8] After the culture has been well started, growth proceeds satisfactorily at room temperature.

Viability of Spirochæta obermeieri.

With the procedure described above, it was possible (1) to cultivate these spirochetes consistently from mouse or rat blood; (2) to maintain the cultures for periods of 3 to 7 weeks; and (3) to carry them on in successive subcultures. In undiluted serum the initial growth is more rapid, but degeneration sets in relatively early (about 2 weeks). In diluted serum or in ascitic fluid, growth is slower, but it extends over a much longer period (7 weeks). Successful subcultures have been obtained from cultures 17, 24, 30, 31, and 49 days respectively after inoculation. As a general routine, the first transplants were made after 12 to 15 days incubation, while in later cultures the interval was 20 to 30 days. A detailed history of some of the cultures is given in Table VI.

SUMMARY.

A study of the growth requirements of *Spirochæta obermeieri* resulted in the perfection of a method which enabled us (1) to cultivate the organisms consistently from the blood of infected mice and rats, (2) to maintain the viability of cultures for periods of at least 3 to 7 weeks, and (3) to carry them on in successive subcultures by transplanting at intervals of 2 to 4 weeks. This method is essentially the same as the Noguchi technique for the cultivation of the *Leptospira* group, but emphasizes control of the physicochemical factors that act to limit and prevent growth and prescribes the conditions necessary to counteract the injurious influences. The main facts may be briefly summarized as follows:

---

[8] The spirochetes grow well at 37°C., but degeneration changes tend to set in early.

(*a*) Ascitic fluid, horse or rabbit serum may be used as culture fluids.

(*b*) These fluids become progressively more alkaline on exposure to air.

(*c*) Uniformly successful results depend chiefly on the proper adjustment and stabilization of the reaction.

(*d*) A balanced reaction can be secured by adding 1.0 per cent peptone broth or egg albumin as buffer, and covering the culture with a layer of oil.

(*e*) The reaction limits for growth and survival are between pH 6.8 and 8.2, with the optimum at pH 7.2 to 7.4.

(*f*) *Spirochæta obermeieri* is a strict aerobe, consequently in order to permit adequate aeration, the oil layer should not exceed 1.5 cm. in height.

THE IMPORTANCE OF DEXTROSE IN THE MEDIUM OF TISSUE CULTURES.

By MARGARET REED LEWIS.

(From the Department of Embryology of Carnegie Institution of Washington, Johns Hopkins Medical School, Baltimore.)

(Received for publication, October 11, 1921.)

Since the metabolism of sugar plays such an important part in sustaining animal life, it seemed of moment to ascertain what influence this substance might have upon cells of tissue cultures which, although living necessarily under more or less anomalous conditions, nevertheless furnish the most satisfactory method known at present for the study of the behavor of cells when exposed directly to any given substance.

Material and Method.

For this investigation over 500 cultures of the connective tissue of chick embryos (7 to 9 days incubation) were prepared in media from which dextrose had been omitted or which contained from 0.25 to 5 per cent of this substance. Various kinds of media were used: white of egg, amniotic or allantoic fluid from chick embryos after 12 or 14 days incubation, Locke's solution containing 10 to 40 per cent bouillon,[1] Locke's solution with from 0.1 to 1 per cent peptone, Locke's solution containing gelatin, Locke's solution with white of egg, and Locke's solution alone.

The cultures were neither bathed nor retransplanted, as the object was to note the effect produced by a given medium without renewing it.

To facilitate the recognition of any abnormal structures that might arise in the cells as a result of other factors in the medium, it was necessary to determine the structure of the normal connective tissue cell. For this, sections of chick embryos and films of fresh embryonic tissue were used.

---

[1] 85 cc. of $NaCl$ 0.9 per cent plus KCl 0.042 per cent plus $CaCl_2$ 0.025 per cent plus $NaHCO_3$ 0.02 per cent plus 15 cc. of chicken bouillon plus dextrose 0.5 per cent is known as Locke-Lewis solution.

Normal Cells.—The connective tissue cell of the young chick embryo was found to be large, irregular, and often spindle-shaped, with a large round or oval nucleus. In some cells the nucleus was bent, in others it was double. The cytoplasm was homogeneous except for numerous rod- and filament-shaped mitochondria, an occasional fat droplet, and in some instances a few minute neutral red bodies. The position of the centrosome was frequently indicated by a clear region near the nucleus. The processes were elongated and usually adherent to those of neighboring cells or to the cover-glass. The cells had the same characteristics in the sections and in the films, provided the films had been prepared in Locke-Lewis solution containing dextrose and were not kept under observation for more than a few minutes. These cells corresponded substantially to those described from sections of the embryo by Duesberg (1910), and in tissue cultures by Lewis and Lewis (1915), Levi (1916), and W. H. Lewis (1919). They did not contain either vacuoles or specific granules.

Abnormal Cells.—When these cells were placed in certain media, other structures made their appearance, some within a few minutes, others not for many days. These abnormalities were for the most part of two kinds: a clear, slightly refractive vacuole and a more or less opaque, rather large granule. In addition to these, there were sometimes giant centrospheres, blebs, liquefaction of the homogeneous cytoplasm, and an accumulation of fat in the cells.

The vacuoles were encountered much more frequently than were the granules. Although never seen in well fixed sections of embryonic tissue, they did form in the cells in film preparations, especially in normal salt solution or Locke's solution lacking dextrose. Prigosen (1921), who made a study of the connective tissue cells of the chick embryo in film preparations, not only describes the accumulation of vacuoles in these cells but points out that certain granules described by other investigators (Albrecht, 1903; Renaut, 1907) are probably also abnormal, in view of the fact that these observers employed the film method. In cultures, however, vacuoles seldom appear before the 2nd or 3rd day. In certain media their appearance was delayed indefinitely, while in others the vacuoles accumulated in the cells until the latter took on a frothy or moth-eaten appearance as described by Maximow (1916) and W. H. Lewis (1919). It is difficult to determine the nature of the vacuoles as they occur under so many conditions; *i.e.*, starvation, degeneration, phagocytosis, lack of oxygen, influence of bacteria, and lack of dextrose. They formed in cells in what would seem to be a most nutritive medium, such as a plasma clot, and were lacking in cells in a medium composed only of salts and dextrose. They certainly seem to indicate the action of some deleterious influence, since, in almost every instance, the accumulation of vacuoles was followed shortly by the death of the cell.

The granules were present much less frequently than were the vacuoles. They never formed in cells in film preparations, but were often observed in cells in plasma cultures and were especially abundant in those grown in white of egg, as was shown in a previous communication (1921). They seemed to be material stored up by the cell attributable to the protein nature of the environment since they were observed only in media containing this substance.

Vacuoles may occur in cells which contain granules and, as previously shown, are especially prone to do so in cells in egg albumin. The granules and vacuoles found in plasma cultures have occasionally been mistaken for different phases of the same body (Maximow, 1916; Shipley, 1919), but this is undoubtedly not always the case, for, as has been shown in regard to cells in egg albumin (1921), the vacuoles may form in regions removed from that occupied by the granules and also, as will appear from these investigations, the formation of vacuoles may be prevented by the addition of dextrose to the medium.

Effects of Lack of Dextrose upon the Cells of Tissue Cultures.

The effect of the lack of dextrose upon the cells of tissue cultures was definite and pronounced, and inevitably resulted in the production of vacuoles. 212 cultures were prepared in various media lacking dextrose, and in every instance vacuoles were formed, after which the cells rapidly degenerated. On the other hand, media such as white of egg, amniotic fluid, and allantoic fluid, otherwise unsatisfactory for cultures, became favorable for the growths when a small amount of dextrose was added.

The lack of dextrose was especially detrimental to cultures grown in Locke's solution or in Locke's solution to which 15 per cent of chicken bouillon had been added. In these solutions without dextrose the cells became full of vacuoles and died within a few days, while in the same solutions containing dextrose they survived many days, depending upon the amount of this substance in the medium. Surprising to say, cells in solutions rich in protein but lacking in dextrose died much sooner than those in a simple salt solution containing dextrose.

Effects of Dextrose upon the Cells of Tissue Cultures.

Only cultures in Locke's solution containing 15 per cent chicken bouillon were used in these experiments, partly because the cells remain more normal in this solution and partly because of the difficulty of attempting to add definite percentages of dextrose to some of the other media.

When only 0.25 per cent dextrose had been added to the medium the cells grew luxuriantly but the formation of vacuoles was delayed for only a few days. The cultures were quite variable. In some the vacuoles developed after 3 or 4 days, while others remained normal for 6 or 8 days. All of the cultures (over 100) in this solution, however, did eventually exhibit vacuoles. These cultures seldom lived more than 10 days. While, as stated above, no attempt was made to add dextrose to the plasma medium, nevertheless it seems apt to mention in this connection that the vacuoles that appear in plasma cultures probably are attributable to the small amount of dextrose (supposedly about 0.25 per cent) which the plasma contains. Cultures that are frequently retransplanted into fresh plasma have a much larger amount of dextrose available and therefore remain normal for longer periods of time.

Cultures in media to which 0.5, 0.75, and 1 per cent dextrose had been added differed little in appearance. The growth in all these media was extensive, full of cell division, and survived from 2 to 4 weeks without forming vacuoles. In some of these preparations, after 2 weeks of healthy growth, the cells one after another rounded up and sank to the bottom of the hanging drop until, within the course of 3 weeks or a month, all the cells had degenerated. These cultures died without forming vacuoles, the last cell being as normal in structure as those of the initial growth. In other preparations, after an interval of healthy growth, all of the cells rapidly formed vacuoles and all degenerated within the following day or two.

When larger quantities (2 to 5 per cent) of dextrose were added to the solution, the growth sometimes was not extensive, and in most instances it did not survive so long as that in the normal solution or in one containing 1 per cent dextrose, but these cells never contained vacuoles. The results obtained when such large quantities of dextrose were added were influenced by the change in hydrogen ion concentration of these cultures.[2] Cultures in normal Locke-Lewis solution, which has an initial hydrogen ion concentration of 6.8 or 7, had usually, upon degeneration, become pH 7.2 or 7.4. Those in media without dextrose had about the same final hydrogen ion concentration. On the other hand, the cultures in media containing as much as 5 per cent dextrose were distinctly acid (pH 5.6 to 6) when tested at degeneration, regardless of the initial hydrogen ion concentration of the medium. The rapidity with which death of the cultures took place seemed to depend upon the change in hydrogen ion concentration. The appearance of these cells after death was usually quite different from that of cells in media containing less dextrose. They did not round up or sink to the bottom of the drop, but remained spread out on the cover-slip, keeping their outlines as though coagulated into skeleton forms.

While the condition which arises in tissue cultures when the amount of dextrose in the medium is varied is somewhat analogous to that in diabetes, it is by no means due to the same cause. The acid which is found in cultures is probably due to the breaking down of dextrose, while the acidosis exhibited by the diabetic is presumably due to the partial combustion of fats resulting in the formation of fatty acids.

---

[2] The details of these results are given in another publication with Felton, who investigated the hydrogen ion concentration of tissue cultures in this laboratory, and for this reason will not be repeated here (Lewis, M. R., and Felton, L. D., The hydrogen-ion concentration of cultures of connective tissue from chick embryos, *Science*, 1921, liv, 636).

SUMMARY.

It is not advisable to enter into a discussion of these findings at the present time, owing to the lack of experimental evidence as to the exact nature of the vacuoles. Regardless of the nature of these bodies or of what factors produce them, they are structures abnormal to the connective tissue cells. The lack of dextrose in the medium of tissue cultures leads to some condition distinctly detrimental to the cells, resulting in their vacuolation and death, even when the medium contains abundant protein material. The addition of small amounts (0.5 to 1 per cent) of dextrose to the medium delays the formation of vacuoles and prolongs the life of the culture. The addition of large amounts (2 to 5 per cent) prevents vacuolation of the cells, but so much dextrose usually leads to a change in the hydrogen ion concentration of the culture resulting in an acid condition which arises coincidentally with the degeneration of the cells.

BIBLIOGRAPHY.

Duesberg, J., Les chondriosomes des cellules embryonnaires du poulet, et leur rôle dans la genèse des myofibrilles avec quelques observations sur le développement des fibres musculaires striées, *Arch. Zellforsch.*, 1909–10, iv, 602.

Levi, G., La costituzione del protoplasma studiata su cellule viventi coltivate "*in vitro*," *Arch. Fisiol.*, 1916, xiv, 101.

Lewis, M. R., Granules in the cells of chick embryos produced by egg albumin in the medium of tissue cultures, *J. Exp. Med.*, 1921, xxxiii, 485.

Lewis, M. R., and Lewis, W. H., Mitochondria (and other cytoplasmic structures) in tissue cultures, *Am. J. Anat.*, 1914–15, xvii, 339.

Lewis, W. H., Degeneration granules and vacuoles in the fibroblasts of chick embryos cultivated *in vitro*, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 81.

Maximow, A., The cultivation of connective tissue of adult mammals *in vitro*, *Russ. Arch. Anat., Histol., et Embryol.*, 1916, i, No. 1.

Prigosen, R., The formation of vacuoles and neutral red granules in connective-tissue cells and blood cells observed under abnormal conditions, *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 206.

Shipley, P. G., The physiological significance of the reaction of tissue cells to vital benzidene dyes, *Am. J. Physiol.*, 1919, xlix, 284.

CONSTRUCTION OF AN OXYGEN CHAMBER FOR THE TREATMENT OF PNEUMONIA.

By WILLIAM C. STADIE, M. D.

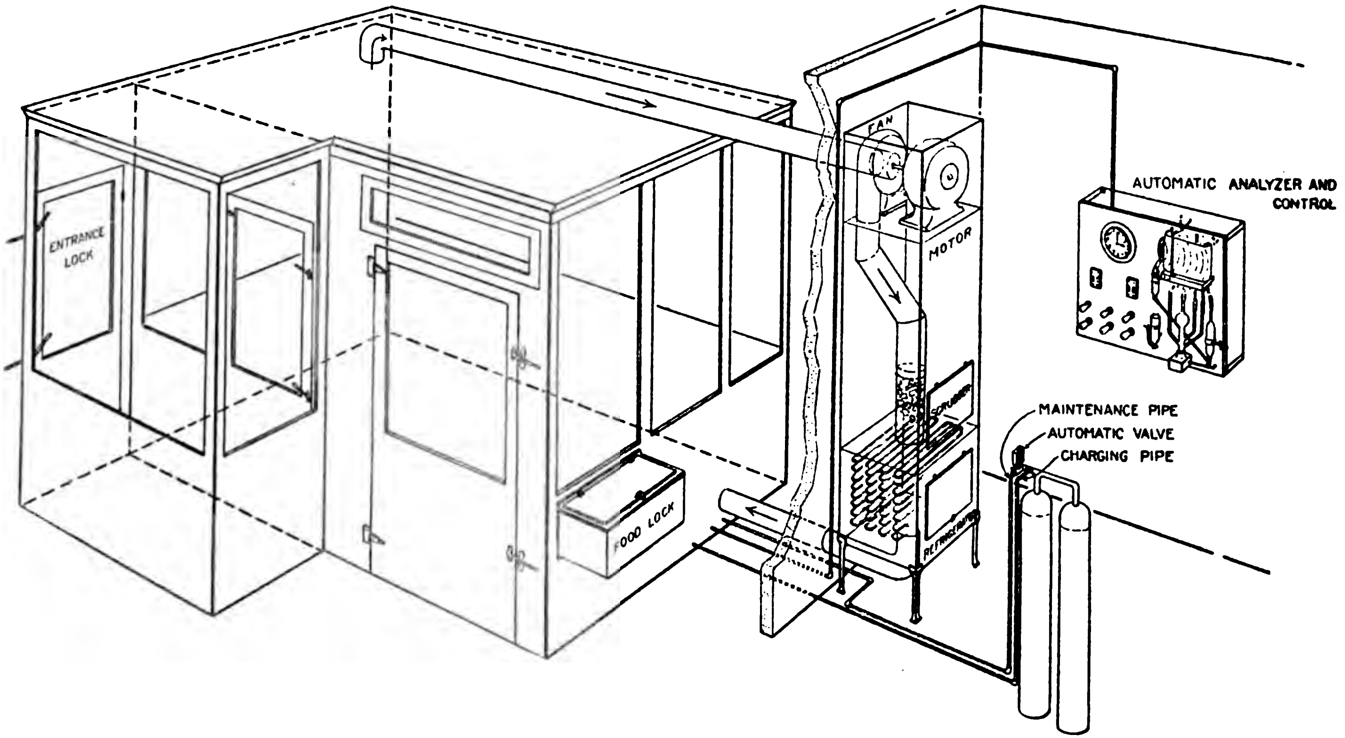
(*From the Hospital of The Rockefeller Institute for Medical Research.*)

PLATE 9.

(Received for publication, October 10, 1921.)

In the construction of a chamber or ward for the treatment of pneumonia patients, which is to be filled with an atmosphere with a higher oxygen content than ordinary air, there are several requirements which must be fulfilled. The chamber must be large enough to accommodate attendants as well as the patient, so that adequate nursing care may be given to acutely ill patients; it must be easily and quickly filled with oxygen to the desired concentration; this concentration must be maintained with facility and a minimum use of oxygen; the temperature of the room must be kept within comfortable limits, and the humidity, which may increase rapidly through the exhalation of moisture from the lungs of the patient and attendants must be kept low; the carbon dioxide excreted must be removed as formed. Moreover, the chamber must be easy of ingress and egress, especially so when acutely ill patients are treated, and above all it must be reasonably economical in the cost of operation. All these objects have been attained in the oxygen chamber described below. It is ready for the routine treatment of patients after a few minutes of preparation and may be run for days with a minimum of attention.

The oxygen chamber (Text-fig. 1 and Fig. 1) has been built in one of the wards of the hospital. It measures 10 × 8 × 8 feet, length, width, and height respectively, and has a total capacity of 640 cubic feet, or 18 cubic meters. The filling, maintenance, cooling, and analyzing devices have all been placed in an adjoining small room for convenience and sightliness. The entire apparatus includes the following: the oxygen chamber proper, doors for entrance, entrance lock, food



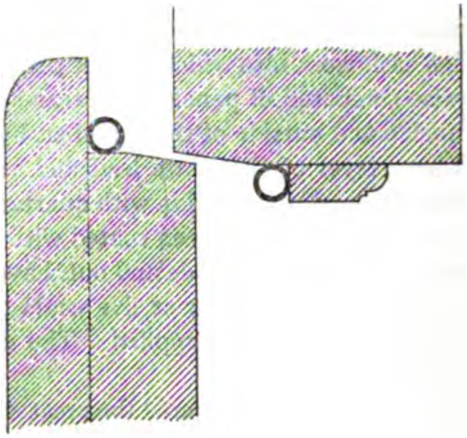
TEXT-FIG. 1. Schematic drawing of oxygen chamber with ventilating system, carbon dioxide scrubber, cooling device, automatic oxygen analyzer, and filling and maintenance devices.

lock, ventilating system consisting of a fan, refrigerator, and carbon dioxide scrubber; the filling and maintenance apparatus consisting of oxygen tanks; a solenoid valve for the automatic regulation of the oxygen; an automatic oxygen analyzer and oxygen maintenance device; a carbon dioxide analyzer.

The chamber proper is built upon the tile floor of the main ward. Special precautions must be taken to insure gas tightness so as to minimize oxygen loss. The foundation consists of a heavy 4 inch base-board which is set upon a flat rubber gasket. Both rubber gasket and base-board are imbedded in a mixture of white lead and varnish, which makes a gas-tight joint with the floor. The lower part of the chamber consists of wooden panels, while the upper part consists of wooden sash work into which are set plate glass windows. The roof is likewise composed of panels. All seams have been covered over, both inside and outside of the chamber, by broad wooden strips set in a heavy layer of the white lead and varnish mixture. In addition, all corners have been filled in with quarter round molding similarly imbedded. The roof has been coated with a heavy pitch, and all grooves and crevices, both inside and out, have been carefully filled with white lead. Over all, four or five coats of paint with a finishing coat of enamel have been applied. The chamber proper is practically gas-tight.

The doors of the chamber have offered the greatest problem with respect to the gas tightness. Reference to Text-fig. 2 will show the type of construction used. A double offset on the door fits closely on similar offsets on the jamb. Two gaskets of soft rubber tubing are placed as shown. When the door is closed, two large refrigerator levers are forced down on the inclined plane of the hooks; a firm and even pressure is thus obtained, bringing both rubber gaskets in close contact with the door, making a practically gas-tight joint. The large door is used for the passage of the patient and the bed into the chamber. It is then closed for the duration of the treatment. Ingress and egress of attendants is made through a smaller door which is protected by an *entrance lock*. The entrance lock, which measures 3 × 8 × 5 feet, with a total capacity of 120 cubic feet, also has a door of the same construction, and serves to minimize the escape of the rich oxygen mixture from the chamber proper into the ward when the doors are opened. The lock, of course, has the same construction as the chamber.

The ventilating system is necessary if the temperature and humidity are to be kept at comfortable levels, and moreover, it gives a movement of the air in the chamber which makes the atmosphere more comfortable. By means of an 8 inch duct leading from the roof, the air in the chamber is drawn out by a *ventilating fan.* From the fan the air is conducted downward through a compartment or *scrubber* which was originally intended to be filled with material for the removal of carbon dioxide. The air then passes through a large *refrigerator* which contains 50 feet of 1 inch piping in which there is an active circulation of cold brine. Here the air is cooled and the excess of moisture precipitated. The air then passes through a 6 inch duct back into the chamber where, to minimize drafts, it is deflected downward toward the floor.



TEXT-FIG. 2. Construction of door showing offsets and rubber gaskets.

Food Lock.—A small *food lock,* measuring 16 × 24 × 13 inches, with a double door fitted with rubber gaskets attached to the side of the chamber is necessary for the passage in and out of the chamber of food and other small articles, thus obviating the necessity of frequent openings of the large entrance lock.

Communication into the chamber is obtained by telephone.

Filling the Chamber.—The oxygen is contained in large steel cylinders under 1,800 pounds pressure. Two tanks are coupled to a manifold or short piece of iron piping, and by means of a reducing valve the pressure is carried down to 0 to 100 pounds pressure. When the chamber is to be filled the oxygen is turned on as rapidly as possible through the reducing valve, and passes directly into the chamber by means of a *charging pipe* ¼ inch in diameter, the fan being run during the filling.[1]

When the charging of the chamber is completed the maintenance of the desired percentage is accomplished automatically. Further admission of oxygen is through the *maintenance pipe* on which is a solenoid *valve* controlled automatically by the oxygen analyzer and automatic regulator.

Automatic Oxygen Analyzer.

This consists of two parts, the analyzer and the electrical controlling device. The operation of the analyzer can be best understood by reference to Text-fig. 3 and Fig. 2 and a description of one complete cycle of operation. The analyzer consists of a gas sampling absorbing

---

[1] It has been found that the filling of the chamber closely follows a curve derived as follows: A vessel whose volume is V, filled with air, has slowly added to it pure oxygen. Diffusion is assumed to occur instantaneously. Excess of the resulting mixture escapes at minute pin-hole leaks, each constituent in proportion to its percentage at the time. If x be the amount of gas (oxygen) added, and y the proportion of oxygen at any time, then if dx of oxygen be added, there escape ydx parts of oxygen and there remain $dx - ydx$.

Therefore, $\frac{dx - ydx}{V} = dy$, the increase in proportion of oxygen.

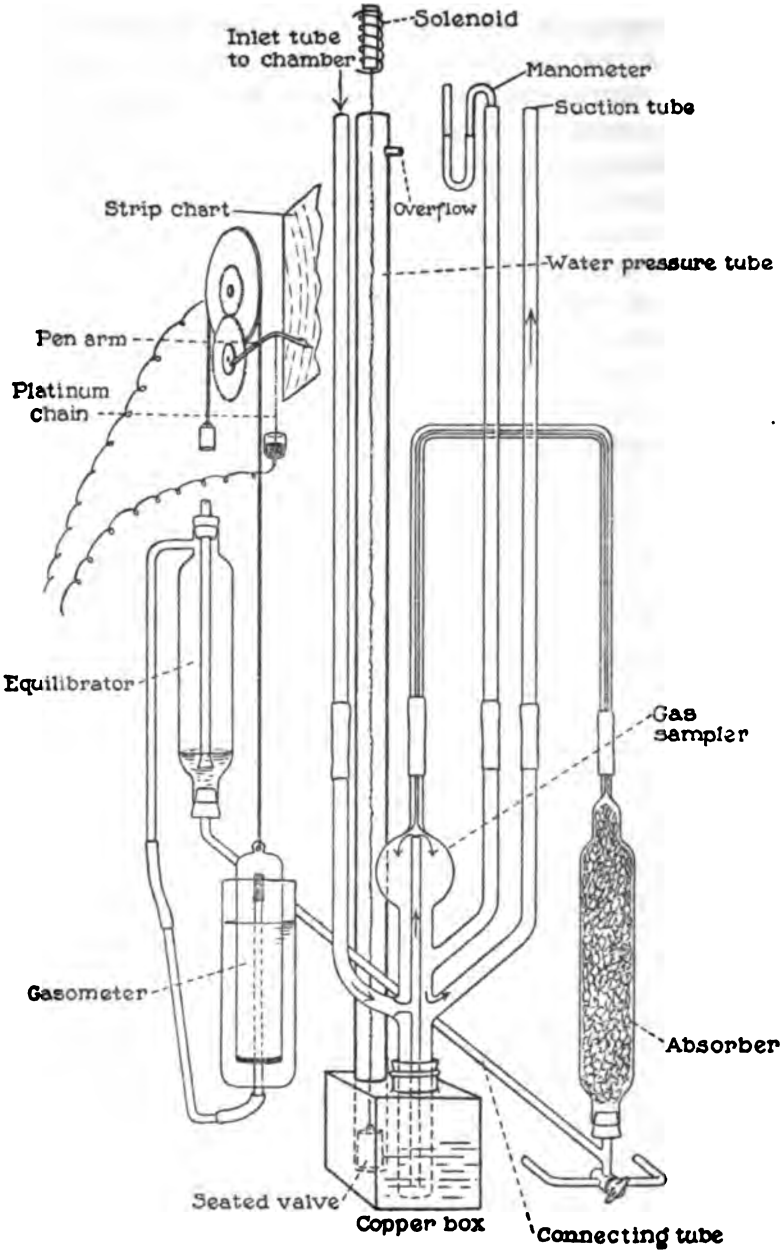
Integrating

$$\int dx = V \int_{y\,=\,0.2\text{ (initial proportion)}}^{y\,=\,\text{proportion desired}} \frac{dy}{1-y}$$

we obtain

$$x = V \operatorname{Log}_e \frac{0.8}{1-y}$$

$$x = 2.303\, V \operatorname{Log}_{10} \frac{0.8}{1-y}$$



TEXT-FIG. 3. Schematic drawing of automatic oxygen analyzer.

bulb, equilibrator, gasometer, meshed wheels, pen arm, and strip chart. The electrical controlling device comprises a clock fitted with contact points and wired to make two contacts every 20 minutes, relays, resistance lamps, and electrical connections to the house current and analyzer. A water vacuum pump is attached to the *suction tube*. By this means a sample of air from the chamber is drawn through the *inlet tube* and the *sampling bulb*. A constant stream of water runs into the *water pressure tube*. The water level slowly rises; when it reaches the height of the inlet and suction tubes, the suction is shut off by the water seal thus formed (the suction now operates through a by-pass, not shown, thus preventing the water and gas sample from being sucked out of the apparatus). A sample of gas is thus trapped in the sampling bulb. The manometer on the manometer tube instantly adjusts the pressure of the gas sample to atmospheric pressure as soon as the suction is shut off by the water seal formed. The water in the water pressure tube and apparatus continues to rise, and the sample of gas in the sampling bulb is pushed over into the *absorber*. The absorber is filled with fine copper wire gauze and a solution of ammonium acetate, ammonium hydroxide, and water.[2] The gas, as it passes into the absorber, displaces an equal amount of the solution which passes up through the connecting tube into the *equilibrator*. A corresponding volume of air in the equilibrator is forced into the *gasometer* which, being counterbalanced, rises and rotates the two meshed wheels, the second wheel carrying a stop-pin which, when the analysis is complete, limits the fall of the *pen arm*. The pen, fastened to a ratchet wheel, is independent of the other two wheels and is held up by a pawl acting on the fine milled ratchet. When the entire sample has been pushed out of the sampling bulb, the water does not rise any higher, but overflows into an *overflow pipe* on the water pressure tube. The gas in the absorbing bulb is allowed to remain in contact with the copper for 20 minutes, during which time all the oxygen is absorbed; and, as a result of this absorption, a certain amount of the solution siphons back into the absorber from the equilibrator. The gasometer sinks

---

[2]

Ammonium acetate	1,000 gm.
Ammonium hydroxide (conc.)	600 cc.
Water to	2,000 cc.

correspondingly. When this absorption is complete, the clock makes the first contact and the small magnet below the gasometer pulls the string which lifts the pawl from the ratchet wheel. The pen then drops until it is stopped by the pin on the small wheel, and its position then corresponds to the amount of oxygen present in the sample. As it falls the pen writes a line on the moving strip chart, the lower point of which indicates upon the scale the percentage of oxygen present in the sample. The second contact is then made by the clock closing the circuit on the *solenoid valve* above the water pressure tube, and this solenoid magnet pulls up a *seated valve* in the copper box, allowing the water in the apparatus to run out. The fluid in the equilibrator then siphons back into the absorber and the gasometer sinks to its original level, rotating the two wheels and bringing the pen to its original position. The valve in the copper box then drops when the contact is broken by the clock, a new sample of gas being meanwhile taken in by the suction, the water again rises in the apparatus, and the operation is repeated.

In addition to analyzing and recording the oxygen percentage the apparatus also controls the solenoid valve on the maintenance pipe to the oxygen chamber above described. The pen carries a small fine *platinum chain* which, through the pen and its support, forms one leg of a circuit, the other leg being connected with the *mercury cup*. The mercury cup is set at any desired position corresponding to the proportion of oxygen desired. When an analysis is completed and the pen drops to record it, if it is less than the desired percentage the end of the platinum chain will dip into the mercury, thus closing the circuit on the solenoid maintenance valve (Text-fig. 1) which opens, allowing oxygen to pass into the chamber. The contacts are so arranged that this valve is kept open for about 2 minutes, during which time from 50 to 100 liters of oxygen may be admitted to the chamber. In this way 300 liters of oxygen may be admitted into the chamber every hour, which is greater than the usual loss. If, however, upon completion of an analysis the percentage indicated is greater than that for which the mercury cup is set, the platinum chain will not touch the mercury and the oxygen valve remains closed. The circuit in the clock and automatic oxygen control carries only currents of a few volts supplied by a battery of lamps from the house current. These contacts control the relays which make and break the 110 volt circuit in the solenoid magnets which have lamp resistances in parallel. In this way heavy current is avoided on light parts and arcing of the contacts is prevented.

With this arrangement it has been found in practice that after the chamber has been charged and the automatic analyzer and regulator started, the oxygen chamber will maintain itself at any desired oxygen percentage from 40 to 60 per cent, with practically no attention except to renew the copper and solution in the analyzer twice a day.

The Removal of Carbon Dioxide.

This offers a serious problem from the point of view of efficiency and economy. It was originally planned to place in the ventilating system, as shown, a container with soda-lime to remove the carbon dioxide as it was formed. It was found, however, difficult to get soda-lime of sufficiently large mesh to offer little resistance to the passage of the air through the system. All of the samples tried, even soda-lime screening from 4 to 6, was found to offer so much resistance as almost completely to shut off ventilation. In practice this resulted in a rapid elevation of the temperature and humidity of the chamber to an uncomfortable degree. Moreover, the best grades of soda-lime are quite expensive. With these two difficulties in mind, it was important to find a substitute. A scrubber was designed for the use of the sodium hydroxide in solution, but this proved very inefficient. The product called natron, which is sodium hydroxide in thin shells, was found to be efficient, easy to handle, and economical. Natron is put out in 3 × 20 inch cylindrical cartons of paraffin cardboard, which have been designed for use in respiratory apparatus and carbon dioxide analyzers of flue gas. One of these cartons is placed on the outlet of a small blower fan which is placed in one corner of the chamber and the air of the chamber is blown through the column of natron. With two people in the chamber one carton will keep the carbon dioxide at practically zero for 2 to 3 hours. The cartons are changed very easily, it being simply necessary to cut out both ends of the carton with a sharp knife and place the fresh one in the blower. In this way the cost of carbon dioxide removal is considerably reduced. Table I illustrates how rapidly and efficiently one natron carton placed on a small Buffalo Forge fan will remove the carbon dioxide.

TABLE I.

Removal of Carbon Dioxide by Carton of Natron.

Time elapsed.	Carbon dioxide.
hrs.	*per cent*
0	1.34
¾	0.05

Water Removal.

In practice the refrigerator has been found quite efficient in keeping the temperature of the chamber down to about 65°F. and also in keeping the humidity between 40 and 50 per cent. Table II shows that even when water vapor is vaporized into the chamber at a rate 20 times the maximum for one person the humidity and temperature are kept within reasonable limits.

TABLE II.

Test of Refrigerator for Removing Water Vapor.

900 gm. of water vaporized into chamber in 1 hour.

Hr.	Time elapsed.	Temperature.	Humidity.
p.m.	*hrs.*	*°F.*	*per cent*
12.30	0	70	64
1.00	½	70	66
1.30	1	71	68
Control; ventilating system not running; office fan in chamber to mix air.			
4.00	0	72	45
4.15	¼	76	53
4.25	½	75	87
5.00	1	76	82

Effect of Opening Doors.

Entrance into the chamber through the lock means, of course, a loss of oxygen. When the inner door is opened into the lock, part of the oxygen from the chamber must pass into the lock. If equilibrium is attained, that is, if there is a rapid diffusion from the chamber into the lock so that the percentage in both is the same, then the decrease of percentage in the main chamber may be calculated from the formula

$$\text{Percentage loss} = \frac{V^1P - 0.2\,V^1}{V + V^1}$$

in which V^1 is the volume of the lock, V is the volume of the chamber, and P is the percentage of oxygen in the chamber. In actual practice the loss is less than this, due no doubt to the fact that when the doors are quickly opened and closed, equilibrium in the lock and chamber with respect to the oxygen percentage is not attained. This is shown in Table III.

TABLE III.

Loss of Oxygen from Oxygen Chamber on Opening Air Lock.

Oxygen in chamber.	Loss of oxygen.			
	Calculated.	Found.	Calculated.	Found.
per cent	*per cent*	*per cent*	*cubic meters*	*cubic meters*
40	3.6	1.1	0.65	0.20

Charging and Maintenance of Oxygen Chamber.

The amount of oxygen required to attain a given percentage of oxygen in the chamber varies, of course, with the percentage desired, and is given by the equation derived on page 327, from which the values in Table IV are calculated. The chamber was maintained usually at 50 per cent of oxygen and 8.6 cubic meters of oxygen are necessary to give this. The amount of oxygen necessary to maintain the oxygen percentage at this figure is small; Table V gives the rate of loss of oxygen in the chamber, ducts, and refrigerator with the ventilating fan running.

In actual practice, however, the loss is greater than this, due to the consumption of oxygen by the patient and nurse, which is about 0.025 cubic meters per hour, and the far greater loss due to the frequent opening of the air lock.

Table VI shows the amount of oxygen required during an actual treatment of a pneumonia patient.

TABLE IV.

Calculated Amount of Oxygen $\left(x = 41.5 \operatorname{Log}_{10} \frac{0.8}{1 - y}\right)$ *Required to Charge Oxygen Chamber to a Given Percentage.*

Oxygen	Volume of oxygen required
per cent	*cubic meters*
30	2.4
40	5.2
50	8.6
60	12.6
70	17.8

TABLE V.

Maintenance Test.

Oxygen chamber charged with 4.9 cubic meters of oxygen. Calculated oxygen = 40.0 per cent. Ventilating fan running. No patient in chamber. Doors not opened.

Time elapsed.	Oxygen.	Loss per hr.	Loss per hr.
hrs.	*per cent*	*per cent*	*cubic meters*
0	39.8		
¼	39.7		
½	39.6		
1	39.2		
3¼	38.7		
13	35.5	0.33	0.060

TABLE VI.

Amount of Oxygen Required for Charging and Maintaining of Oxygen Chamber during Treatment of Pneumonia Patient.

Patient.	Duration of treatment.	Oxygen maintained.	Amount of oxygen required to charge chamber.	Amount of oxygen required for maintenance.	Loss per hr.
	hrs.	*per cent*	*cubic meters*	*cubic meters*	*cubic meters*
U.	24	40–50	9.3	4.0	0.17
U.	17	50–60	9.4	2.0	0.14
Total......	41		18.7	6.0	0.31
			Grand total........ 24.7		

SUMMARY.

1. The construction of an oxygen chamber is given. This chamber can be quickly filled with oxygen to any concentration up to 65 per cent and maintained at the desired concentration for an indefinite time.

2. The construction of ventilating system, cooling device, carbon dioxide remover, automatic oxygen analyzer, and filling and maintenance devices is given.

3. The chamber is designed so that pneumonia patients with anoxemia may be placed in it and breathe an atmosphere containing 40 to 60 per cent of oxygen.

4. The chamber is easy of ingress and egress, is economical in cost of operation, and comfortably accomodates patient and attendants so that adequate nursing and medical attention can be given at all times.

EXPLANATION OF PLATE 9.

Fig. 1. Photograph of oxygen chamber in use to show size relative to patient and attendants.

Fig. 2. Photograph of automatic oxygen analyzer with electrical controlling device.

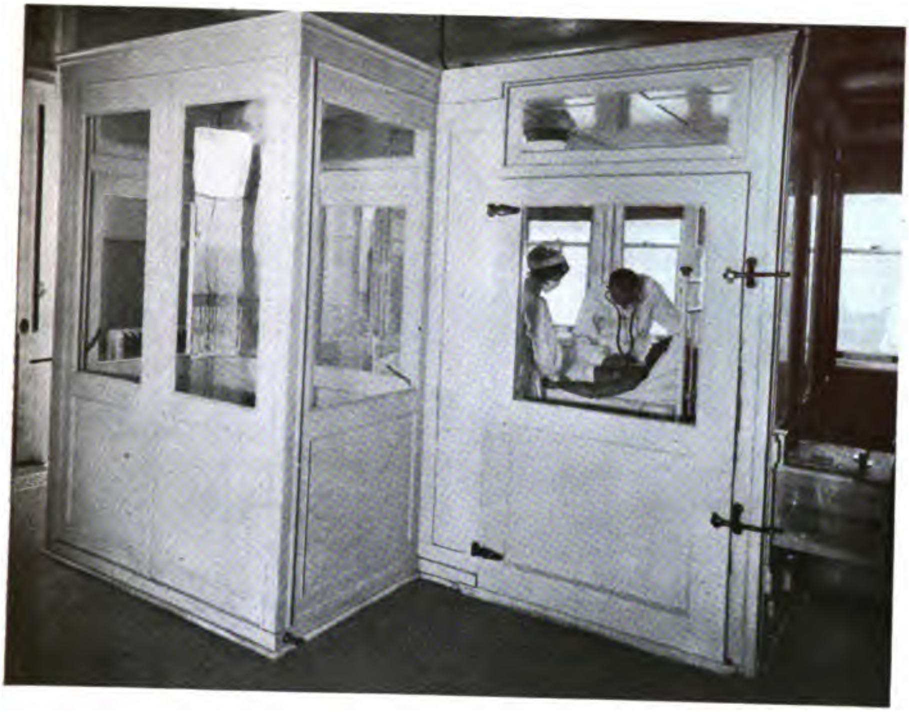


FIG. 1.

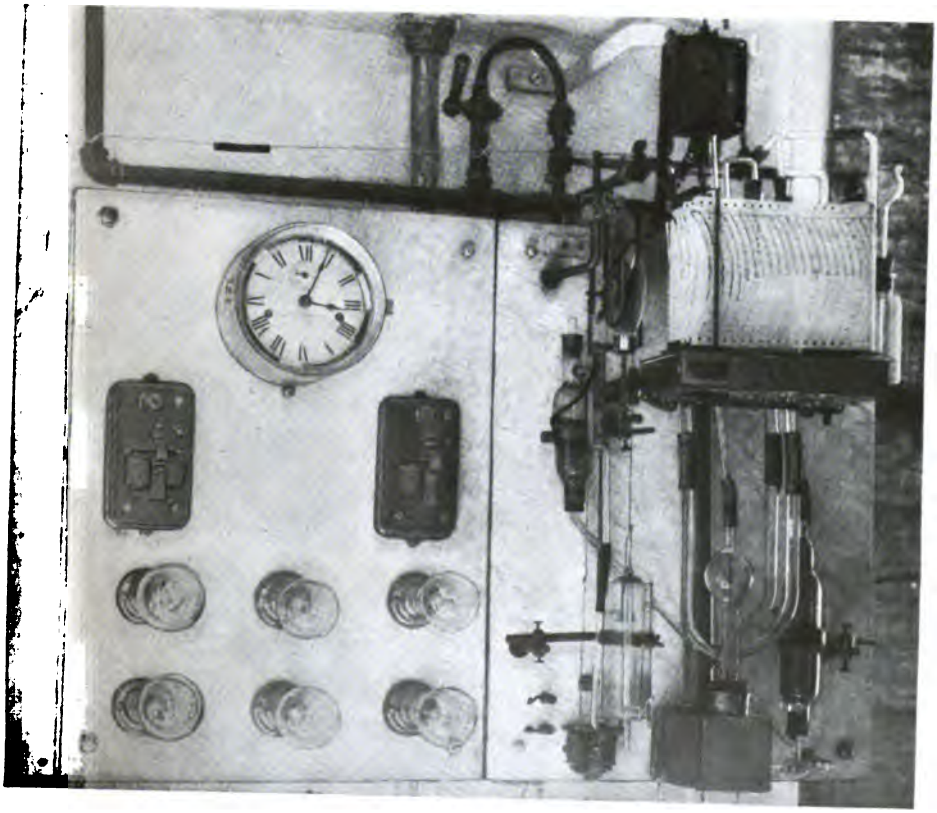


FIG. 2.

(Stadie: Oxygen chamber for treatment of pneumonia.)

THE TREATMENT OF ANOXEMIA IN PNEUMONIA IN AN OXYGEN CHAMBER.

By WILLIAM C. STADIE, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 10, 1921.)

Shortly after the discovery of oxygen, and particularly after the demonstration by Lavoisier of oxidation as a life process, oxygen came into use as a therapeutic agent. It has been given by inhalation, by subcutaneous and intravenous injection (Stuertz, 1903; Tunnicliffe and Stebbing, 1916), and by rectal insufflation. In addition, ozone also has been extensively used by inhalation, and hydrogen peroxide given intravenously in cases of influenzal pneumonia (Oliver and Murphy, 1920). Oxygen itself has been used in the treatment of a great variety of diseases; the literature which has grown up about its use is extensive; the opinions regarding its efficacy and the conditions under which it should be used are varied. Little would be gained by a review of this literature here; nevertheless, it is important to state the reasons for its use in disease, giving particular emphasis to the therapeutic use of oxygen in the treatment of pneumonia patients.

The use of oxygen as a therapeutic agent is rational only when, by reason of a disturbed metabolism or an insufficient oxygen supply, either local or general, there exists a condition of suboxidation. This may be either acute or chronic, and presumably results in harm by virtue of the fact that intracellular processes are disturbed by diminished oxidation or accumulations of unoxidized products of cell activity. There are many causes of suboxidation, but the one which interests us chiefly here is commonly called anoxemia. The term anoxemia as ordinarily used means "hypo-oxemia," or a condition in which there is a diminished amount of oxygen in the circulating blood; hence anoxemia is more correctly defined as that condition in which the hemoglobin of the blood is less saturated with oxygen than normally. It must not be assumed that anoxemia necessarily presupposes a condition of suboxidation, for the relations between normal function and function under varying degrees of anoxemia are not definitely established. It will suffice to point out here why it is usually assumed that anoxemia results in a condition of suboxidation and is therefore deleterious to the organism.

Oxygen Tension of the Blood and Tissue Tension.

In considering the passage of oxygen from the arterial blood to the tissues two factors must be recognized: normal blood has available for tissue respiration about 20 volumes per cent of oxygen (capacity factor) at a tension (intensity factor) ranging from 100 to 0 mm. Hg. The average normal amount of oxygen taken by the tissues from the arterial blood is 6 volumes per cent, or 30 per cent of the total capacity. The dissociation curve of average human blood (Barcroft, 1914) shows that when the arterial blood is completely saturated, the 6 volumes per cent will be delivered to the tissues at a tension greater than 35 mm. Hg. In other words, the amount of oxygen ordinarily used is available at this relatively high tension. The remaining 16 volumes must be given to the tissues at relatively low tension (less than 35 mm. Hg.).

If, however, the blood in the arteries is only partially, *e.g.* 70 per cent, saturated, there are still available for the tissues 14 volumes per cent of oxygen. This is more than enough for ordinary purposes. But this oxygen is at a tension less than 35 mm. Hg. A person with this degree of anoxemia is in extreme distress. Although the oxygen of his blood is abundant in amount, it is available at low pressures only; so that unless it is assumed that the tension at which the oxygen is available for tissue metabolism is of as much importance as the total amount, it is difficult to understand how such a condition of anoxemia can be harmful.

There is but little and inconclusive evidence, however, to show that the tension of oxygen in the blood in contradistinction to the total amount available is important. Verzár (1912) and later Krogh (1919) have demonstrated that, contrary to previous conceptions, the oxygen tension of the tissues is not zero, but between 30 and 40 mm. Hg.; this would indicate that the tissues ordinarily *do* not, although it does not show that they *may* not, function at low oxygen tensions. Vernon (1906) has studied metabolism of tissues under varying oxygen tensions, and his results, while far from conclusive, seem to indicate that tissue metabolism is not normal when the oxygen tension falls below a certain minimum. Verzár (1912), on the other hand, obtained contradictory results on different tissues, although his results on muscle, the functionally simplest tissue he used, showed that there is a striking decrease in oxygen consumption at low blood oxygen tensions. Burrows (1917) likewise, in studying the growth of tissue cells, concluded that whereas high oxygen tension is without influence, growth does not occur when the oxygen tension falls below that corresponding to the average oxygen tension of the venous blood of mammals.

The condition of anoxemia, resulting from carbon monoxide poisoning, is explainable also as due to a diminished tension of the blood oxygen. Presumably carbon monoxide itself has no poisonous action, and its harmful effects are due entirely to the temporary destruction of the oxygen-carrying power of the blood. This by itself would be difficult to understand, for saturation of as little as 50 per cent of the blood with carbon monoxide is serious, and saturation of 80 per cent of the hemoglobin is always fatal. Yet, in the first case there would still be in the average individual about 10 volumes per cent of oxygen available, and in the second case there would still be 4 volumes of oxygen available, which, as Lundsgaard (1919) has shown in cases of pernicious anemia, is about the lowest possible value compatible with life. Haldane (1912), however, has shown that in blood but partially saturated with carbon monoxide the oxygen dissociation curve of the available hemoglobin is markedly modified. The initial half of the curve is much steeper and the second half much flatter than normally, so that at low tension of oxygen the blood is more saturated with oxygen than normal blood, but, conversely, holds on to its oxygen more tenaciously in the capillaries. Reference to the curve will show that in a blood half saturated with CO only 2 volumes per cent of the available 10 volumes of oxygen will be at a tension above 50 mm.; the remaining oxygen will be at a lower partial pressure. From this Haldane concludes that anoxemia of carbon monoxide poisoning is due to a marked diminution of the tension of the oxygen of the blood.

Although this experimental evidence is far from conclusive, clinical experience gives suggestive support to it. Congenital cardiac disease is associated with chronic anoxemia, and it is possible that the mental and physical deterioration seen in these cases is due in part at least to anoxemia. One case under our observation had an oxygen capacity of 25 volumes per cent, the arterial blood being 80 per cent saturated. Despite the 20 volumes per cent of available oxygen in the arterial blood, typical signs of physical and mental deterioration were present. It would be difficult to understand how anoxemia could play any rôle in this condition unless the tension of oxygen in the blood is important.

In this connection, however, we must consider the fact that under certain conditions the body may use all the oxygen in the blood. In other words, the oxygen, even at the lowest tension, is utilizable. When the arm is exposed to cold the venous blood may contain little or no oxygen (Meakins, 1921). Again, Lundsgaard (1919) showed that in cases of pernicious anemia in which the oxygen capacity was as low as 5 to 6 volumes per cent the venous blood from the arm was almost completely deprived of oxygen, and he concluded that: "The last residuum of the oxygen in the blood . . . . is taken away by the body cells in resting individuals just as easily as the first part." In the postmortem heart's blood of pneumonia cases Stadie (1919) found the blood as little as 5 per cent saturated.

On the other hand, while certain tissues may use oxygen at low tensions it does not follow that their function is normal, and it may well be that other tissues, particularly the kidney and the central nervous system, show disturbed function with deficient oxygen tension. Lutz and Schneider (1919) and Ellis (1919) showed that the respiratory center is quite sensitive to comparatively slight changes in oxygen tension, and recently Doi (1921) has confirmed this in cats. Haldane (1919) in experiments upon himself showed marked mental disturbances under an oxygen tension of 40 mm. His arterial blood would be then about 75 per cent saturated and still had 13.8 volumes per cent of blood oxygen available. This amount, while more than normally necessary, was at a low tension, and hence presumably not available for the central nervous system. Meakins and Davies (1920) report an experiment in which the subject by breathing low oxygen mixtures reduced the arterial saturation to 81.4 per cent. Headache and general malaise resulted.

In striking contrast to the distress which follows anoxemia when the oxygen in the blood is abundant but at low partial pressures are cases of anemia. With as little as 6 to 8 volumes of oxygen per 100 cc. of blood these patients are quite comfortable at rest (Lundsgaard, 1919). The blood here is completely saturated, and hence the oxygen, while small in amount, is at a high pressure.

A well known response to anoxemia is an increase in the oxygen capacity of the blood. This is observed in pneumonia with cyanosis, congenital and acquired cardiac disease, as well as after exposure to high altitudes. In this way the amount of oxygen supplied at all tensions is increased. At any given percentage saturation the blood will give up more oxygen than normal blood so that this mechanism compensates in part for the partial saturation of the blood in the arteries.

Use of Oxygen in Pneumonia.

In pneumonia there frequently occurs a condition of anoxemia. While there is no direct evidence to show that this acute anoxemia, often of profound degree, is harmful, nevertheless it is usually assumed that the presence of anoxemia is dangerous. In a series of thirty-three pneumonia cases (Stadie, 1919) there was only one case which recovered in which the arterial unsaturation of the blood was greater than 20 per cent. A high degree of anoxemia in pneumonia, then, is accompanied by a high mortality, and yet it must be distinctly remembered that they are not necessarily cause and effect, since the degree of anoxemia varies directly with the severity of the infection and the extent of the consolidation. It is possible then that the anoxemia is simply a concomitant feature of intense and extensive infections and plays no rôle in the ultimate fatality. Not until the relation of function to oxygen tension is further elaborated can it be definitely said that an anoxemia *per se* is a factor in the fatal outcome.

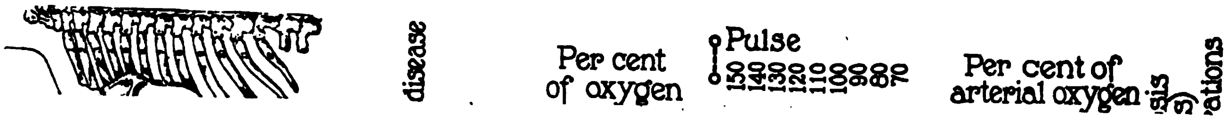
Since anoxemia is a frequent and often a pronounced symptom of pneumonia, a study of the effects of oxygen upon this type of anoxemia and upon the course of the pneumonia was begun and is here reported. The anoxemia is due to an insufficient aeration of the blood in its passage through the lungs. As to the mechanism of this deficient aeration, it is usually assumed that the consolidation of part of the lung, the presence of many small patches of infiltration extending from the main or initial focus, the plugging of many small bronchi, and the coating of the alveoli with exudate and moisture diminish the respiratory surface or hinder the diffusion inward of oxygen. This explanation does not stand alone, and recently Meakins (1920, *a*) states that: "The anoxemia occurring in acute lobar pneumonia is the result of the rapid and shallow breathing typical of this condition" which purely mechanically lessens ventilation of the alveolar spaces. In both cases the administration of oxygen would tend to relieve the anoxemia by greatly increasing the percentage of oxygen in the alveolar air and hence its diffusion pressure.

In critically ill cases of pneumonia, then, it is conceivable that anoxemia might make serious inroads upon the resistance of the patient and hasten the end. Certainly experience has shown that cases with an arterial unsaturation greater than 20 per cent usually proceed to a rapid and fatal termination. In these cases the relief of anoxemia might prolong life until the forces of immunity could assert themselves.

The cases here reported were treated with oxygen in an oxygen chamber described in the accompanying paper (Stadie, 1922). By this means the treatment could be continued over a prolonged period of time, the percentage of oxygen administered fixed at any desired level, discomfort and inconvenience of masks, funnels, and tubes eliminated, adequate medical and nursing attention rendered at all times, and, moreover, accurate observations of the blood and respiration made under exactly known conditions. Eight cases are here reported. All had a high degree of anoxemia and presented grave prognoses.

EXPERIMENTAL.

Eight cases from the pneumonia service of the Hospital of The Rockefeller Institute were treated from 1920–21. The cases treated were selected from all the pneumonia cases during this time. The mode of selection was as follows: All cases were carefully followed as to the degree of anoxemia present from time to time. The presence


disease
Per cent
of oxygen
Pulse
150
140
130
120
110
100
90
80
70
Per cent of
arterial oxygen
sis
ations

and extent of the anoxemia were estimated by determination of the arterial unsaturation. The extent of cyanosis, particularly in the finger-tips, also furnishes a valuable guide. When a case developed anoxemia of sufficient degree, usually 20 per cent or more of arterial unsaturation, oxygen treatment was commenced. The cases were followed by repeated determinations of the arterial unsaturation. When it was not feasible to do arterial punctures, estimates of the degree of anoxemia were made from the extent of the cyanosis. In order to compare cyanosis from time to time the following scale was used: ± indicates very slight, + slight, ++ moderate, +++ marked, and ++++ intense cyanosis. Experience enables an observer to estimate closely, in this way, the degree of arterial unsaturation, as controlled by actual blood analysis. The case reports follow.

Case 1.—D. G., male, age 25 years. Admitted Mar. 25, 1921.

Present Illness.—Onset Mar. 21, with severe abdominal pain, nausea and vomiting, fever, headache, prostration, and chills.

Physical Examination.—Temperature 104.5°F.; pulse 160; respirations 36. No cyanosis. The condition of the lungs is shown in Text-fig. 1. Rest of physical examination negative.

Mar. 28. Patient is quite toxic. Has developed hiccough. Lung signs are as diagramed in Text-fig. 1. In addition to the area of consolidation on the right side there are many fine râles on the left side. Pulse rather poor in quality. Cyanosis +. 2 p.m. Patient is much worse than this morning. Cyanosis +++. Arterial unsaturation 37 per cent. There are many moist râles involving both sides. Oxygen treatment started.

Mar. 29. Patient's condition much improved. He is still delirious. Has continual hiccough. Cyanosis absent. Arterial unsaturation 3 per cent. Lung signs about the same as yesterday. 80 cc. of sterile fluid withdrawn from right chest.

Mar. 30. During his stay in the oxygen chamber the patient appeared much improved. There were extra systoles. Removed from the chamber last night, since which time he has been becoming worse. Respirations more rapid and irregular; hiccough at every respiration. Cyanosis has increased markedly. Arterial unsaturation 22 per cent. Evidence of consolidation of entire right side. There are coarse moist râles throughout both lungs. 4.30 p.m. Patient is distinctly worse and the outlook is very doubtful. Cyanosis is extreme. Arterial unsaturation about 35 to 40 per cent, estimated from degree of cyanosis. Oxygen treatment commenced. 10 p.m. Cyanosis has entirely cleared up. Hiccoughs continue and are extremely prostrating.

Mar. 31. No cyanosis. Arterial unsaturation 1.5 per cent. All efforts to relieve hiccough without avail.

Apr. 1. Lung signs as shown in Text-fig. 1. Hiccough still continues. No cyanosis.

Apr. 3. Temperature is almost normal. Cyanosis +. Hiccoughs still persist.

Apr. 5. Patient stopped hiccoughing quite suddenly. He had hiccoughed without interruption for 9 days. For this reason he was not taken out of the oxygen chamber sooner. Convalescence uninterrupted.

Bacteriological Examination.—Type II pneumococcus recovered from the sputum. Blood cultures negative.

Discussion.—When the impairment of the respiratory surface had extended to the opposite side of the initial lesion, as shown by the presence of râles, cyanosis became much more marked. The anoxemia disappeared under the effect of oxygen. A short period without oxygen during the course of the illness resulted in a return of the marked anoxemia, so that the patient's condition looked quite desperate. This anoxemia was again relieved by oxygen. Recovery followed.

Case 2.—E. K., male, age 25 years. Admitted Feb. 3, 1920.

Present Illness.—Acute onset 1 week ago with malaise, headache, body pains and cough, and blood-tinged sputum.

Physical Examination.—Negative except for lungs. Slight dullness at left lower back. No râles. No cyanosis.

Feb. 4. Lung signs as diagramed in Text-fig. 2. No cyanosis.

Feb. 6. Definite signs of consolidation of entire right lower lobe. Many coarse musical râles on the right side. Patient has a definite cyanosi (+).

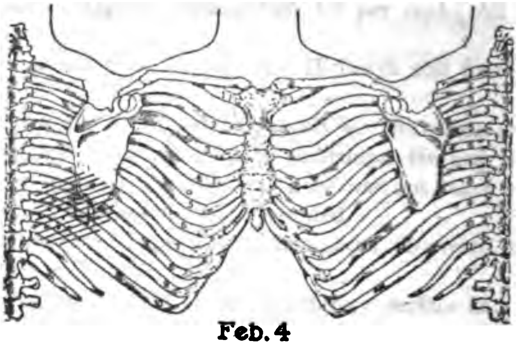
Feb. 7. The patient is definitely worse this morning. Cyanosis is more marked. Patient delirious. Lung signs are as diagramed in Text-fig. 2. Arterial unsaturation 22 per cent. Patient is extremely ill and prognosis is grave. Oxygen treatment started.

Feb. 8. Patient has been in the oxygen chamber since yesterday at 4.30 p.m. Cyanosis is distinctly less. Arterial unsaturation has decreased to 5 per cent. Lung signs about the same as yesterday.

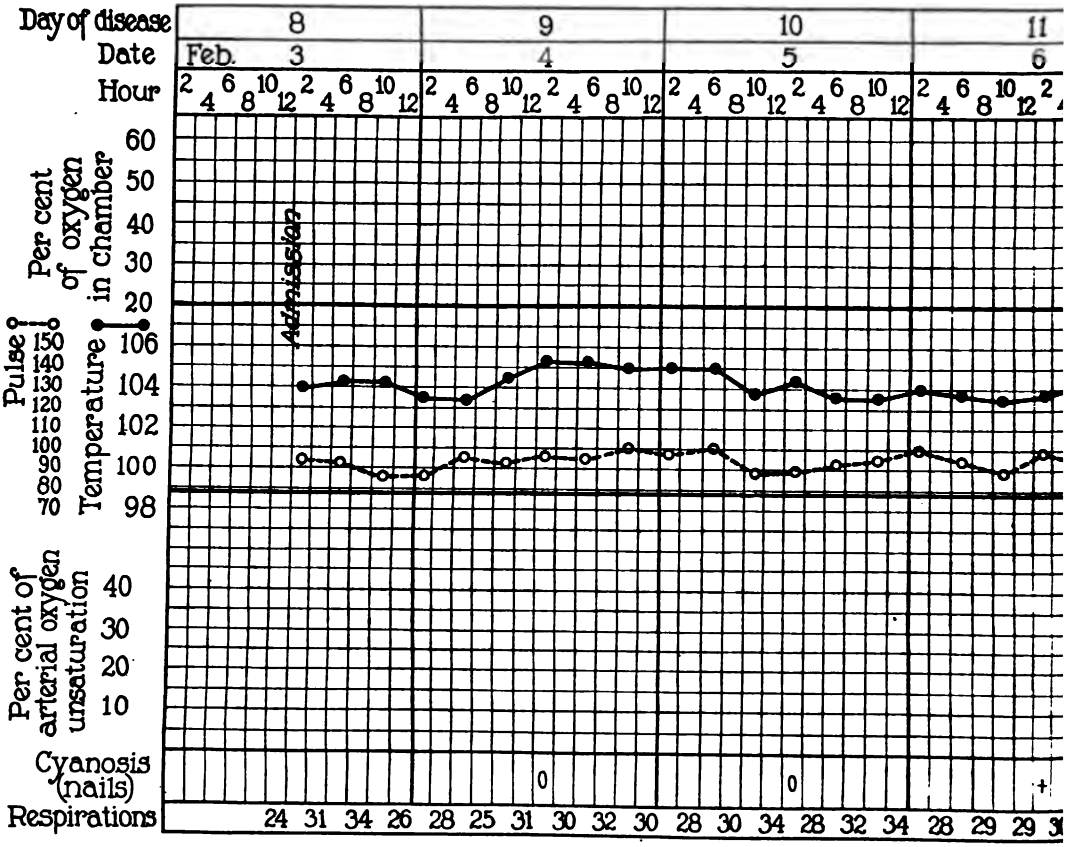
Feb. 9. Condition much better than on Feb. 7. Cyanosis has disappeared. Mentally he is more alert. Signs are as indicated in Text-fig. 2. Patient was removed from the oxygen chamber at 3 a.m. but soon relapsed into a critical condition. Cyanosis gradually returned until at 2 p.m. there was marked (+ + +) cyanosis and arterial unsaturation had increased to 35 per cent. The patient looks desperately ill. Oxygen treatment recommenced.

Feb. 10. Patient had marked jaundice. Lung signs much the same as yesterday. Cyanosis has entirely disappeared.

Feb. 11. Patient's condition much improved. The lung signs have cleared up in that extensive râles on the right and left sides have disappeared, there being signs of consolidation of the right lower lobe only. Oxygen treatment discontinued. No cyanosis. Arterial unsaturation 5 per cent. Convalescence uneventful.



Feb. 4


Day of disease
8
9
10
11
Date
Feb. 3
4
5
6
Hour
2 4 6 8 10 12 2 4 6 8 10 12
Per cent of oxygen in chamber
60
50
40
30
20
Admission
Pulse
Temperature
150
140
130
120
110
100
90
80
70
106
104
102
100
98
Per cent of arterial oxygen unsaturation
40
30
20
10
Cyanosis (nails)
0
0
+
Respirations
24 31 34 26 28 25 31 30 32 30 28 30 34 28 32 34 28 29 29

Bacteriological Examination.—Type IV pneumococcus recovered from the sputum. Blood cultures negative.

Discussion.—At first the patient had slight signs which greatly increased until there was consolidation of the entire right lower lobe. Patient became more and more toxic. Cyanosis developed as lung signs increased, and with the appearance of extensive moist râles throughout both lungs, anoxemia became marked. Anoxemia was completely relieved by the administration of 40 per cent of oxygen. When the oxygen was withdrawn while the patient still showed evidence of extensive impairment of the lungs, his condition became much worse and the anoxemia advanced rapidly to 36 per cent. Readministration of oxygen relieved the anoxemia completely. Recovery followed.

Case 3.—H. B., male, age 41 years. Admitted Apr. 29, 1920. Chief complaint pain in chest and cough; duration 6 days.

Present Illness.—Onset Apr. 23, with sharp pain in the chest and chill, and cough with blood-tinged sputum. Patient delirious for the past 24 hours.

Physical Examination.—Patient quite sick. Marked cyanosis. Arterial unsaturation 20 per cent. Lung signs as diagramed in Text-fig. 3. Extensive consolidation of left side.

Apr. 30. Signs practically the same as yesterday. Cyanosis and arterial unsaturation the same.

May 1. Patient is definitely worse this morning. Spread of lung signs as shown in Text-fig. 3. Cyanosis (+++) more marked. Arterial unsaturation has increased to 25 per cent. Oxygen treatment started at 12 noon. 4 p.m. Cyanosis now only + ; arterial unsaturation 12 per cent. 12 midnight. Patient is now pink, in striking contrast with the deep purplish cyanosis of this morning.

May 2. No cyanosis. Arterial unsaturation 6 per cent. Patient looks and feels much better. Temperature decreased to normal. Oxygen treatment discontinued. Convalescence uneventful.

Bacteriological Examination.—Type II pneumococcus from the sputum. Blood cultures negative.

Discussion.—Patient had extensive infection, with anoxemia lasting for a long period of time. Signs involving the entire left side with moist râles. Cyanosis became much more marked (+++). Treatment with oxygen caused rapid disappearance of cyanosis and anoxemia. Condition rapidly became normal.

Case 4.—J. V. S., male, colored, age 20 years. Admitted Mar. 4, 1921. Chief complaint cough and pain in the side.

Present Illness.—The day before admission the patient had a severe chill, headache, and developed a cough and bloody sputum. There was severe pain in the right side.

Physical Examination.—Negative except for lungs. Left lung normal. Right lung: Dullness from the fourth rib to the base; bronchial breathing with fine moist râles.

White blood corpuscles 20,600; polymorphonuclears 92 per cent.

Mar. 7. The patient ran an even course up to today. About + cyanosis and 15 per cent arterial unsaturation (Text-fig. 4). Today he is much sicker. Cyanosis has increased (+++). Arterial unsaturation has advanced to 35 per cent. Lungs show an extensive consolidation which involves the entire right lower lobe.

Mar. 8. Patient extremely toxic. Respiration rapid and labored. There is ++ cyanosis and 20 per cent arterial unsaturation. Patient is distinctly worse than yesterday, respirations being as high as 50 to 60. Oxygen treatment started at 12 noon. By evening the cyanosis had disappeared and arterial unsaturation had dropped to 5 per cent.

Mar. 9. Patient is somewhat better than yesterday. There are signs indicating extension of the process to the left lower base. No cyanosis. Arterial unsaturation 7 per cent. 8 p.m. Patient's temperature has receded, and although he still appears quite ill oxygen treatment is discontinued. The patient ran a prolonged course with evidence of consolidation of both lower lobes. Although the toxemia disappeared in great measure, the fever continued and the temperature did not reach normal until the 25th day. Convalescence then was uninterrupted.

Bacteriological Examination.—Pneumococcus Type II from the sputum. Blood culture negative.

Discussion.—The patient had a pronounced degree of anoxemia for a considerable period of time, at one time as high as 35 per cent of arterial unsaturation. Simultaneously with the spread of the pneumonia to the opposite side the patient became sicker. Under the influence of oxygen the anoxemia cleared up and concomitantly there was a fall in temperature and diminution in the severity of the toxic symptoms.

Case 5.—G. F., female, colored, age 17 years. Admitted Feb. 15, 1921. Chief complaint headache and pain in left side.

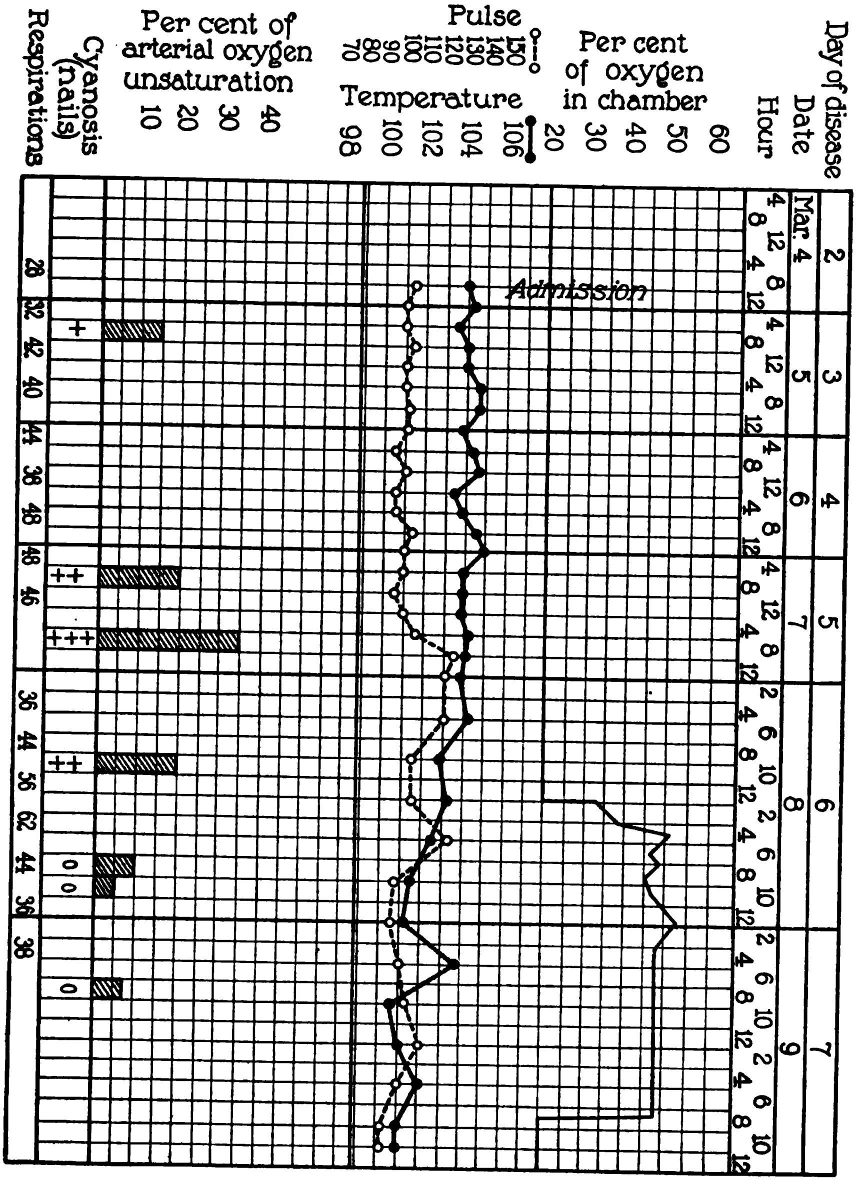
Present Illness.—Onset Nov. 9, 1920, with severe headache and cough, and pains in the left chest followed by nausea and vomiting. Scanty sputum.

Physical Examination.—Temperature 104.5°F.; pulse 140; respirations 50. Lungs: Dullness on the left side from the fourth rib to the base. Over this area there is bronchial breathing. Scattered râles over both lungs.

Feb. 17. Patient has been quite delirious during the night. Definite consolidation of the left lower lobe. Scattered râles throughout both lungs. Cyanosis ++ with 22 per cent arterial unsaturation (Text-fig. 5).

Feb. 18. The patient is still quite sick. Cyanosis (+++) has increased and arterial unsaturation is about 25 to 30 per cent estimated. 4 p.m. Oxygen treatment started.

Feb. 19. Oxygen treatment continued. Cyanosis has disappeared. Lungs: Complete consolidation over the entire left side with scattered moist râles both front and back. Scattered fine râles over right side. No cyanosis during the day.



TEXT-FIG. 4. Case 4.

Feb. 20. Patient is still extremely toxic. Lung signs as yesterday with scattered fine and medium sized moist râles over both sides. No cyanosis.

Feb. 21. The patient had a very good night and temperature is receding. Oxygen treatment discontinued yesterday afternoon. Patient had profuse perspiration and temperature fell by crisis. Convalescence uninterrupted.

Bacteriological Examination.—Type II pneumococcus was recovered from the patient's blood.

Discussion.—This was an intensely toxic patient with a process involving the entire left side. Considerable degree of anoxemia. The anoxemia was relieved by the oxygen which had no other apparent effect upon the course of the disease.

Case 6.—H. S., male, age 59 years. Admitted Mar. 1, 1921. Chief complaint cough and fever; duration 2 days.

Present Illness.—Up to time of onset the patient was feeling well. Feb. 26, 1921. He had a sudden attack of severe pain in the left side and then developed a severe cough productive of large amounts of thick, yellow sputum.

Physical Examination.—Temperature 103° F.; pulse 100; respirations 28. Lungs: Right side clear. Left lung: Dullness over the upper lobe; exaggerated breath sounds anteriorly. Scattered medium moist râles over left upper lobe. No cyanosis.

White blood corpuscles 24,000; polymorphonuclears 92 per cent.

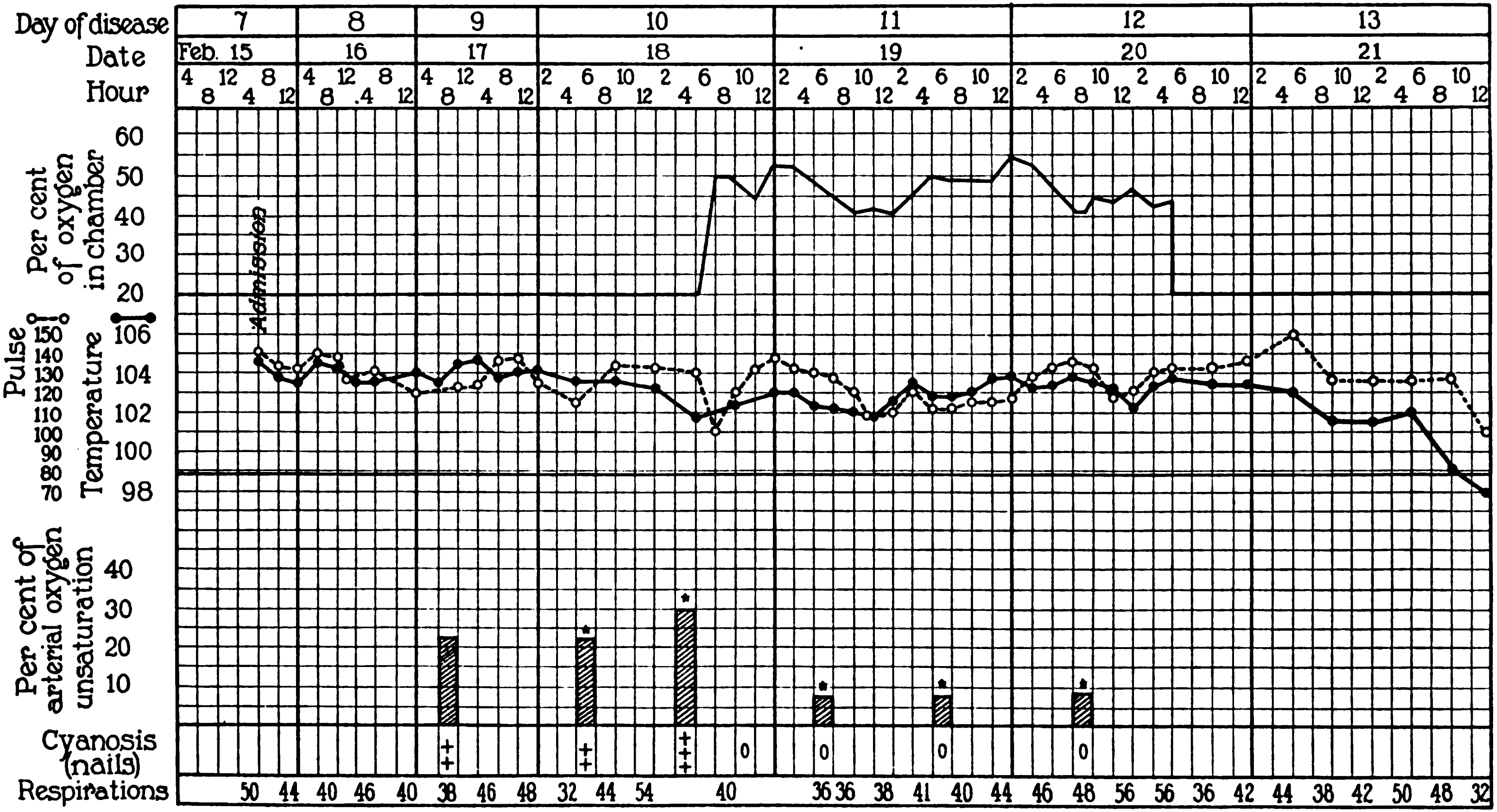
Mar. 2. Patient toxic. Cyanosis +. Consolidation over left upper lobe with scattered moist râles. 12 midnight. Patient is more toxic than this morning. Cyanosis the same (+).

Mar. 3, 6.30 a.m. Patient had a sudden attack of edema of the lungs; respirations rapid; pulse about 160, barely perceptible. Respirations labored. Coarse bubbling râles heard throughout the entire chest (Text-fig. 6). Patient deeply cyanotic (++++), cyanosis involving the entire hand, and face being very dusky; semicomatose. Condition grave. 9.30 a.m. Patient has been in the oxygen chamber since 6.30 a.m. His condition is still grave. Cyanosis (++) has cleared up in part; arterial unsaturation 10 per cent. There are still coarse râles throughout both lungs. 11.45 p.m. Patient's progress has been down hill despite the administration of 60 per cent of oxygen. Cyanosis (+++) distinctly increased. Lungs have rapidly filled up until everywhere there are large, coarse, bubbling râles. Pulse of poor quality during the day. 11.48 p.m. Died.

Bacteriological Examination.—Pneumococcus Type II from the sputum. Blood culture negative.

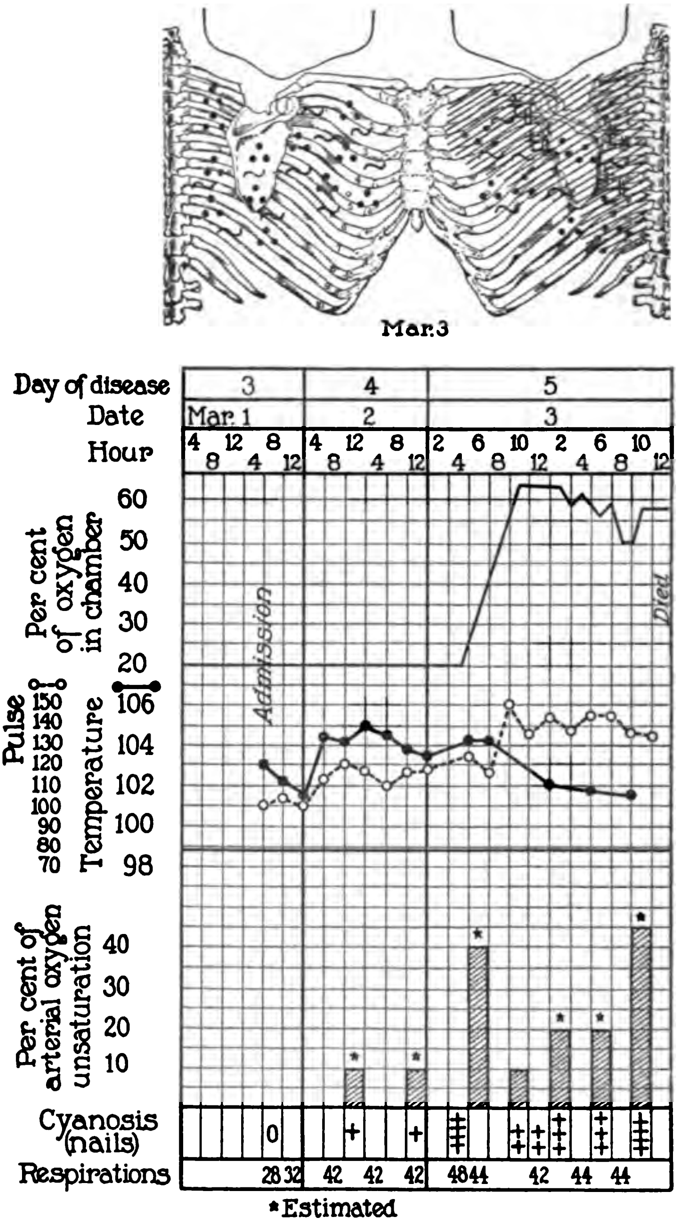
Autopsy.—Extensive tuberculosis involving the entire left lung, with cavity at the apex. At the right apex there was a small area of tuberculous infiltration. Both lungs showed extensive edema and many small patches of bronchopneumonia.

Discussion.—On account of the extensive tuberculosis the outlook in this case was, of course, hopeless. The sudden attack of edema of the lungs with the impairment of respiratory surface was accompanied by great cyanosis which

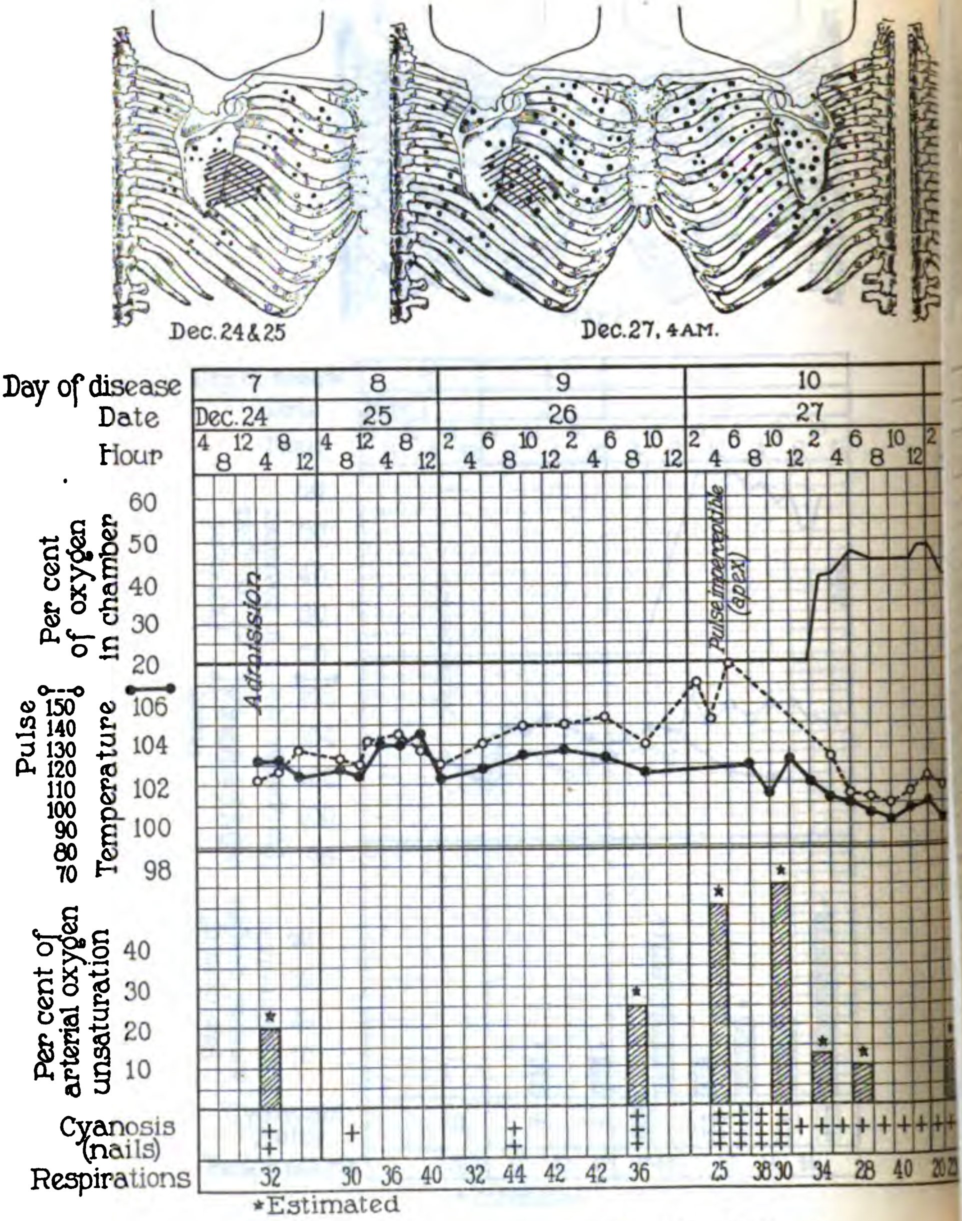


* Estimated

TEXT-FIG. 5. Case 5.

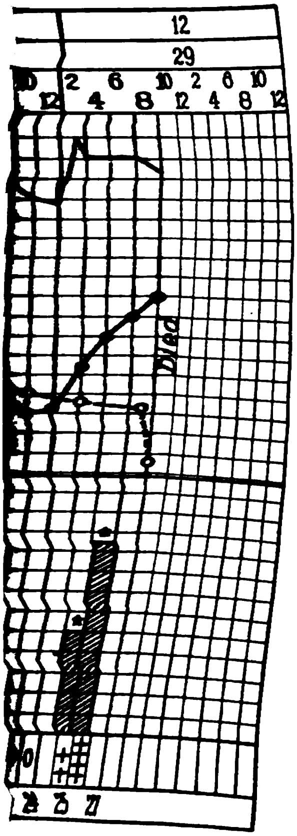


TEXT-FIG. 6. Case 6.



TEXT-FIG. 7. Case 7.

Dec. 29



ieved by the administration of high oxygen. In the face of ıa the oxygen was subsequently without avail in relieving the

emale, age 42 years. Admitted Dec. 24, 1920. Chief com- ack and cough.

—The patient has been treated at the tuberculosis clinic at ›spital for 5 or 6 years. All during this time she has had râles ıgs. No definite consolidation. Tubercle bacilli have never sputum. Tentative diagnosis was tuberculosis.

Onset Dec. 21, 1920, with severe backache, weakness, pros- Temperature 103–104°F.

ation.—Patient restless, toxic. Temperature 103.6°; pulse Lungs: Anteroposteriorly on both sides extensive medium Posteriorly at the fifth rib dullness and harsh breathing. osis with estimated arterial unsaturation of 20 per cent.

's condition is graver. Pulse poor in quality. Respirations increased until it is about +++ with estimated arterial › 25 per cent.

Patient's condition is critical. She is pulseless and deeply Breathing gasping and difficult. Heart rate estimated at). Condition looked almost hopeless. Respirations rapid ing and spasmodic. Patient comatose. Lungs: All over noist râles, fine, medium, and coarse (Text-fig. 7). 12 noon. worse this morning. Extreme cyanosis (++++). Esti- uration 50 to 60 per cent. Patient pulseless. Respirations p.m. Patient put in oxygen chamber with 50 per cent of time cyanosis had disappeared, respirations became more ›ing up to 36, and had lost the gasping quality. In a few rned to radial artery and had fallen to 100. Temperature vas rational and presented the most remarkable contrast to norning.

›nt has been kept in the oxygen chamber with a percentage at 12, when it was dropped to 40 per cent. Patient very time. Pulse continues good. Respirations have dropped red, but deep and regular. Practically no cyanosis (+). aturation 5 to 10 per cent. Lung signs about the same as -day, except that râles are much less numerous. Over the right lower lobe there are definite signs of consolidation. lition as far as temperature, pulse, and respirations were that the oxygen treatment was discontinued. Oxygen was vly and at 4 p.m. the doors were opened. 6.30 p.m. The .e; pulseless; breathing gasping in character; lungs showed over; cyanosis ++++. Oxygen was again started and rapidly run up to 40 per cent and later to 60 per cent. There was temporary relief of the condition, cyanosis disappearing completely.

Dec. 29. The increased oxygen undoubtedly tided her over the night. The improvement, however, did not last long. Patient soon became absolutely pulseless, extremities cold, comatose, had extreme jaundice, and died at 7 p.m.

Bacteriological Examination.—From the sputum was recovered a Pneumococcus Type IV. No tubercle bacilli found.

Discussion.—Patient had an old pulmonary history which suggested tuberculosis, although there was no definite proof of this. The case was remarkable in that with the definite spread of the initial consolidation an extensive involvement of the lungs was manifested by many fine and coarse râles on both sides. Patient became extremely anoxemic and her condition quite serious. Administration of oxygen resulted in remarkable improvement in the condition which, however, was only temporary. No autopsy.

Case 8.—T. R., male, age 60 years. Admitted Jan. 4, 1921. Chief complaint pain in the right ear, headache, pain in the chest, cough, and expectoration of bloody sputum.

Present Illness.—Onset Jan. 3, with great prostration, and pain in the back and chest with severe cough.

Physical Examination.—Friction rub in right axilla; fine moist râles at right side anteriorly. Small area of dullness in posterior axillary line from the fifth to the seventh ribs with bronchial breathing. Left side clear. Cyanosis +.

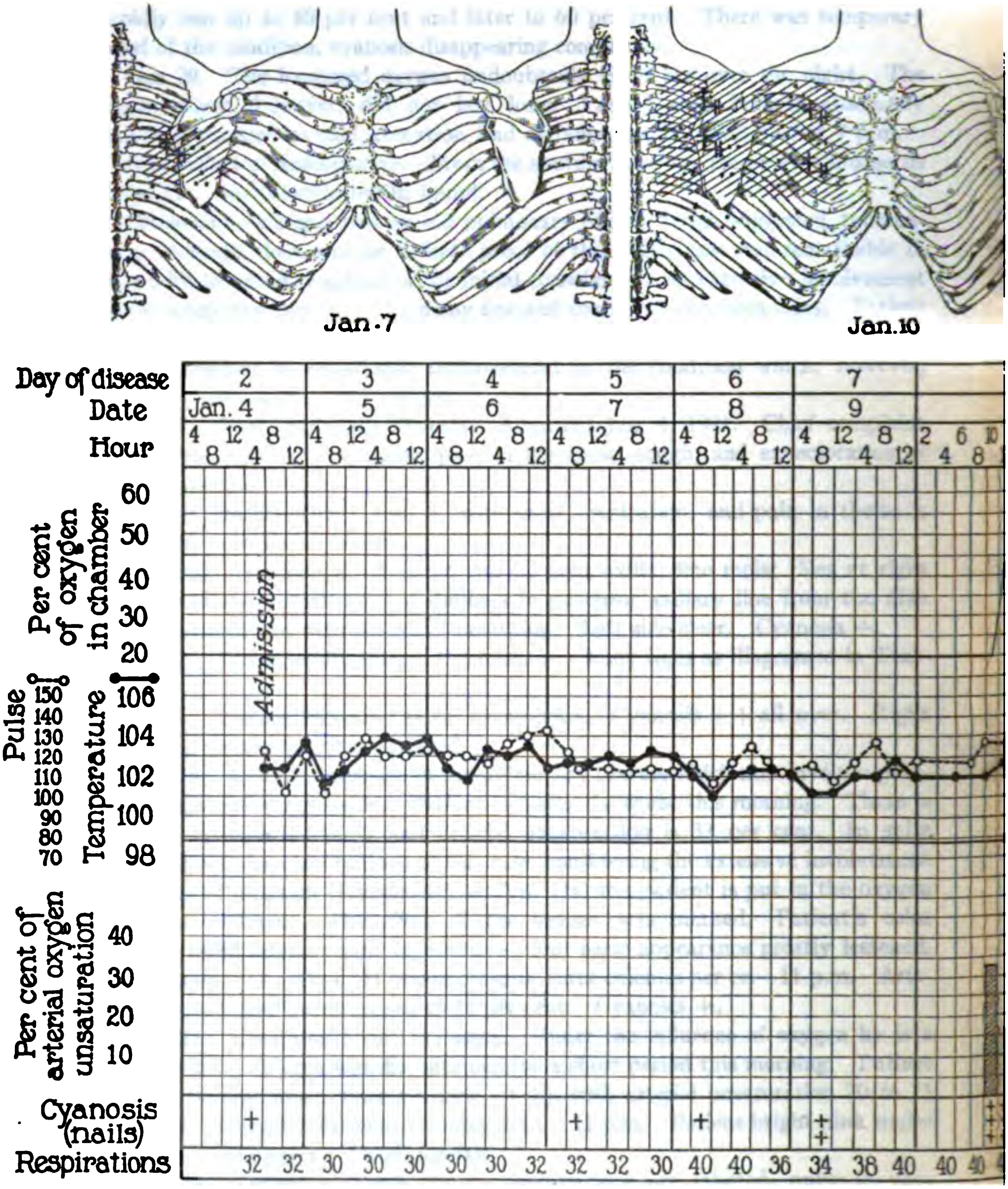
Jan. 7. Patient quite toxic. Cyanosis +. Lung signs as diagramed in Text-fig. 8.

Jan. 9. Patient becoming sicker every hour. Cyanosis ++ all over. Right ear drum incised and pus obtained.

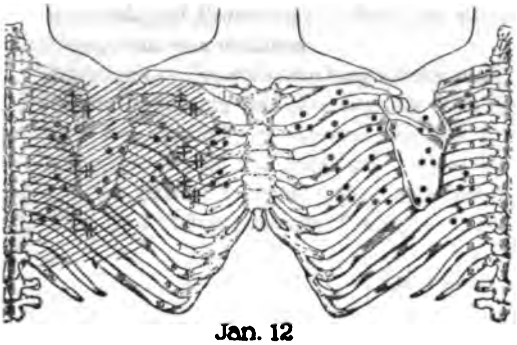
Jan. 10. Definite extension of the pulmonary process which now involves practically the entire right side. Patient is distinctly worse this morning. There is marked cyanosis (+++) and arterial unsaturation is 33 per cent. In spite of the almost hopeless outlook of this case, considering the extensive involvement and type of infection (Pneumococcus Type III) the patient is put in the oxygen chamber. 10 p.m. The effect of the oxygen was marked. Patient's color showed rapid improvement (cyanosis +) and toxic appearance greatly lessened. Blood culture + ; Type III pneumococcus, three colonies per cc. 11 p.m. Arterial unsaturation has decreased to 16 per cent. Cyanosis +.

Jan. 11. Condition about the same. Under the influence of oxygen he is a bright pink. Oxygen was discontinued for a short period this morning. Patient rapidly became much more cyanotic (++), with arterial unsaturation 20 to 25 per cent. Oxygen treatment recommenced. 11 p.m. Patient bright pink under 60 per cent of oxygen. Condition fair.

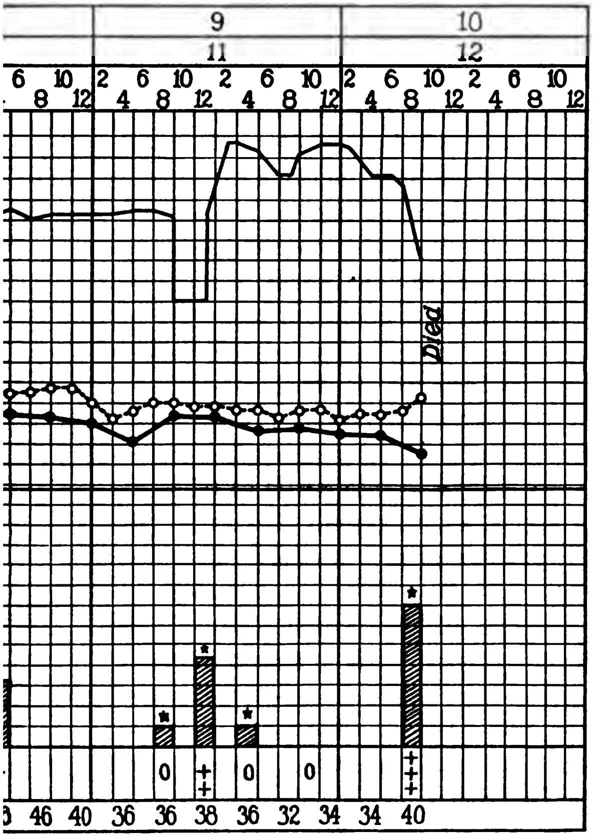
Jan. 12. Patient is much worse. Respirations are labored, pulse is very feeble, and he is markedly cyanotic (++++) despite 60 per cent of oxygen. Examination of the lungs shows extensive râles involving both lungs, with coarse bubbling râles all over. 8 a.m. Died.



Text-Fig. 8. Case 8.



Jan. 12



* Estimated

ype III

rganism
t of the
ll as to
discon-
porarily

is now
t even
y cases
oxemia
ecomes
accom-

d. By
iffusion
hrough
. This
rial un-
e blood
oxemia
inistra-
mained
ht pink
us pur-

anoxe-
nber of
ared in
neasure
ression,
lement,
d grave
s given
oxygen

chamber he became rapidly worse and it was the opinion of all who saw him then that his chances of recovery were slight. The immediate improvement under oxygen for the second time was striking. Case 2 showed the same improvement on the first administration of oxygen. In a short time after removal from the oxygen chamber the patient was in great distress. The cyanosis was +++, the arterial unsaturation 35 per cent, the breathing difficult and labored. There was beginning jaundice which later became intense. There was every indication that the patient was dying. The change in the general condition of the patient shortly after the second administration of oxygen was as remarkable as in the previous case. Case 7 gave a convincing demonstration of the distinctly beneficial action of oxygen in an anoxemic pneumonia patient. The patient, obviously dying, was pulseless, the heart rate was 160 to 170, there was deep cyanosis, the extremities were cold, her respirations gasping, and she was comatose. The lungs were filled everywhere with coarse, bubbling râles. Yet within a short time after the oxygen treatment was begun the condition had changed completely. Then the patient was bright pink, the pulse had returned to the radial artery, and it was strong (120), respirations were quieter, and she was mentally alert. The fact that the patient died finally makes the demonstration of the action of oxygen more convincing in as far as the immediate relief of grave symptoms is concerned. The remaining cases likewise showed in general the same changes in the clinical picture following oxygen.

These patients are, then, examples of critical cases of pneumonia which sooner or later present a picture of severe toxemia upon which is added a marked disturbance of the oxygen transfer from lungs to tissues. They are thus struggling against infection with a marked handicap, and our experience has shown that these anoxemic patients do not do well. In the last analysis recovery will depend upon the specific resistances to the infection which the patient can muster, but while oxygen can in no sense be specific, the above cases strongly indicate that marked improvement follows its administration and presumably life was prolonged. In five of the eight cases recovery ensued. Ordinarily the recovery of these cases was extremely doubtful.

There were three fatal cases. Of these, one (Case 6) had, as shown at autopsy, extensive tuberculosis upon which was superimposed a fresh pneumonia. Case 7 had been treated at the Presbyterian Hospital for years for a chronic bronchitis and was a suspected tubercular case although no definite proof of this was found. The third fatal case (No. 8) was a man of 60. The type of infecting organism (Type III pneumococcus) present in the blood and lungs is known to be highly virulent and the mortality in cases with a positive blood culture is close to 100 per cent.

On the whole, the series here reported, though small, is encouraging. The relief of anoxemia by oxygen appears to be beneficial.

It would perhaps be better to commence oxygen treatment as soon as anoxemia is observed. Any of its harmful effects could then be prevented.

In order that oxygen treatment in pneumonia should be of benefit it must be given over a long period of time. It is futile to give oxygen haphazardly at infrequent intervals, and by methods which are wasteful. Moreover, the percentage of oxygen which reaches the lungs should be approximately known and controlled. The inhalation for a long time of 100 per cent of oxygen is known to be injurious (Smith, 1899), and unless oxygen is given by properly controlled methods, it is possible that the alveolar air may be almost pure oxygen. An oxygen chamber is the method of choice. The patient suffers no added discomfort, and can receive nursing and medical attention as if in the open ward. Moreover, a definite percentage of oxygen can be given. Accurate observation of the course of the disease can be made under known conditions.

Other devices may be used. The Haldane (1917) mask is efficacious and economical. Hill (1921) has devised a bed tent for use in oxygen therapy. Meltzer (1917) used his insufflation method in pneumonia and reported striking results. With these and other devices which may be easily constructed the further study of the action of oxygen as a therapeutic measure can be made. The essential features of such devices should be ease and economy of operation, adequate removal of carbon dioxide, moisture, and heat, and comfort for the patient with as little interference with the medical and nursing care as possible. Most important of all, the device must enable oxygen to be given over a long period of time and the percentage of oxygen must be easily controlled.

Mode of Production of Anoxemia in Pneumonia.

A study of cyanosis and anoxemia in relation to lung signs in a large series of cases has shown that the extent of the cyanosis depends upon the amount and kind of involvement. This becomes evident when careful daily plotting of lung signs is done together with observations of the anoxemia from the arterial unsaturation and cyanosis. A few typical examples are given in the lung charts accompanying the case reports. As a rule when the pneumonic process is confined to one area, as in the typical lobar pneumonia variety, there is little or no cyanosis. The physical signs, *viz.* dullness, bronchial breathing, and a few fine râles, are then confined to the area involved. Kline and Winternitz (1915) and later Gross (1919) have shown that in this type of consolidation there is little or no circulation of blood to the consolidated lung, and therefore little or no admixture of non-aerated with aerated blood occurs. When, however, there is a spread of the pneumonic process, and there occur scattered and secondary areas of bronchopneumonia, the blood circulation in these areas is intact. Moreover, there is a more or less widespread bronchitis with considerable peribronchial pneumonia and edema. The lung signs are changed. In addition to the original area of dullness and bronchial breathing, there are other areas of impaired resonance and harsh breathing. Also râles are heard over a considerable part of the lung, sometimes on one side, often on both. These râles are medium sized or coarse, moist râles, and indicate a considerable involvement of the lungs with edema, consolidation, and pneumonic infiltration. Many of the alveoli are thus functionless, others are coated with exudate, bronchioles are wholly or partially occluded, and the efficient ventilation of the lungs is greatly hindered. Anoxemia results.

A study of the lung signs in the case reports shows that anoxemia is proportional to the extent of respiratory surface involved. In Case 1 on March 25 there was a complete consolidation of the right lower lobe. There was no cyanosis. On March 28 and 29 there was a definite spread of the initial process and many fine râles were present on the opposite side. The anoxemia was now 37 per cent with a

+++ cyanosis. On March 30 the process was still more extensive, coarse moist râles were heard on both sides, and without oxygen the arterial unsaturation rose to 30 to 40 per cent (estimated) with a ++++ cyanosis. The moist râles persisted for a number of days and during that time even under increased oxygen some anoxemia was present.

Case 2 more strikingly illustrates this. On February 4 there were signs of consolidation over the right lower lobe only, with no cyanosis. On February 7 the consolidation had extended and a marked impairment of the respiratory surface was indicated by the presence of numerous coarse râles on both sides posteriorly. There were now a ++ cyanosis and 22 per cent of arterial unsaturation. On February 9, when oxygen was withdrawn, anoxemia reappeared (35 per cent of arterial unsaturation). There were still many coarse râles on both sides. On February 11 the râles had disappeared, leaving only the initial area of frank consolidation. The cyanosis was +, the arterial unsaturation normal.

In Case 3, on April 29 and 30, with signs of consolidation over the entire left back, the anoxemia was considerable (cyanosis ++; arterial unsaturation 20 per cent). On May 1 the involvement was more extensive, with coarse râles all over the left side. The cyanosis (+++) and arterial unsaturation (25 per cent) were definitely increased.

In Case 6, on March 1 there was no cyanosis. The lung signs (not charted) were dullness and bronchial breathing over the left upper lobe with fine moist râles. On March 3 the signs had spread, involving the entire left lung. There were numerous coarse râles on both sides. The cyanosis was ++++.

In Case 7, with an increase in the extent of pulmonary involvement as indicated by an increase in the number of râles, the anoxemia definitely increased.

In Case 8, on January 7 there was slight anoxemia (cyanosis +) with a small area of consolidation and scattered râles over the right upper lobe. On January 10 the entire upper and middle lobes showed dullness and bronchial breathing. There were coarse râles over the entire right side. Anoxemia was marked (arterial unsaturation 37 per cent; cyanosis +++).

These protocols show definitely that a spread of the pneumonitis, as indicated by dullness, bronchial or harsh breathing, and especially the presence of edema as indicated by râles, is associated with increase in the anoxemia. Whether the amount of pneumonitis, bronchitis, and edema is the sole factor in the production of anoxemia is at present difficult to state.

Haldane, Meakins, and Priestley (1919) studied rapid and shallow breathing in normal subjects. In some cases they produced periodic breathing, but only in one experiment was cyanosis observed. In this case the subject was breathing at the rate of 156 per minute, the tidal air being 159 cc. In other experiments the respiratory rate varied from 69 to 150 per minute and the tidal air was 69 to 161 cc. No cyanosis was reported in these cases even when the tidal air was as low as 69 cc. No blood analyses were done. Haldane, Meakins, and Priestley conclude: "that the shallow breathing causes uneven ventilation of the lungs and this in turn produces anoxemia and consequently periodic respiration and other symptoms." Meakins and Davies (1920) repeated this work with analysis of the arterial blood. In the one experiment reported they found that a change in the tidal air from 586 to 315 cc. produced a diminution of the arterial saturation of 2.6 per cent. On the basis of this and the previous experiments with Haldane and Priestley, Meakins (1920, *b*) concludes that rapid and shallow breathing produces anoxemia in pneumonia, and he reports four cases in which the tidal air and extent of cyanosis were studied. In these cases he found that with a decrease in the tidal air there was an increase in the cyanosis. No protocols of lung signs are given. Later (1921) he states that: "the degree of pulmonary consolidation is of less importance than the type of interference with respiratory function" in the production of anoxemia.

The extremely rapid and shallow type of breathing studied by Haldane, Meakins, and Priestley is, of course, quite outside of any observed range in health or disease. In studies of many cases of pneumonia I have rarely observed a tidal air below 250 cc. Moreover, cyanosis was apparently a rare occurrence even with this extreme type of shallow breathing. In the experiment of Meakins and Davies (1920) the tidal air was cut in half. This is much more like the condition met with in pneumonia. The diminution of the arterial saturation was slight, only 2.6 per cent, or about 0.5 volume per cent in average blood. This is in striking contrast to the 20 to 40 per cent of arterial unsaturation frequently observed in pneumonia patients whose tidal air ranges between 250 and 350 cc. While it is possible that the type of breathing may be responsible in some measure for the production of anoxemia, it is difficult with the evidence at hand to see how it can be the sole or even the most important cause of the great anoxemia often associated with pneumonia.

SUMMARY.

1. The use of an oxygen chamber in the treatment of pneumonia patients makes it possible to administer this gas for long periods of time under exactly known conditions. The medical and nursing care of the patient is greatly facilitated.
2. Prolonged inhalation of oxygen varying from 40 to 60 per cent appears to be without harm.
3. Oxygen administered to intensely anoxemic patients almost immediately clears up this anoxemia. Cyanosis disappears with the anoxemia.
4. The removal of patients from the high oxygen while they are still sick and while examination shows that there are still extensive edema and infiltration of the lung results in a return of the intense anoxemia.
5. It is sometimes impossible to clear up the anoxemia, even when as high as 60 per cent of oxygen is given, especially when there are considerable edema and infiltration of the lungs.
6. Five cases in which the prognosis was grave recovered. Three cases, one of tuberculosis, one with a Pneumococcus Type III infection, and a third with a pneumonia superimposed on a chronic pulmonary condition, died.
7. In all cases there appeared to be an improvement in the patient's condition. In one case, particularly, with an intense degree of anoxemia, the patient became moribund and pulseless. Following the administration of 60 per cent of oxygen there was a lowering of the heart rate from 160 to 120, the return of the pulse to the radial artery, the color became bright pink, and there was a remarkable change in the clinical condition.
8. The anoxemia of pneumonia is due, in large measure, to an impairment of the respiratory surface of the lungs. The greater the lung involvement the greater the anoxemia. Especially is this so

when the pneumonic process extends throughout the lungs so that there are many patches of bronchopneumonia, with accompanying bronchitis and edema, as evidenced by the presence of râles throughout the lungs.

9. Rapid and shallow breathing of the degree observed in pneumonia is, as far as present evidence shows, of less importance in the production of anoxemia.

BIBLIOGRAPHY.

Barcroft, J., Respiratory function of the blood, Cambridge, 1914, 226.
Burrows, M. T., *Am. J. Physiol.*, 1917, xliii, 13.
Doi, Y., *J. Physiol.*, 1921, lv, 43.
Ellis, M. M., *Am. J. Physiol.*, 1919–20, l, 267.
Gross, L., *Canad. Med. Assn. J.*, 1919, ix, 632.
Haldane, J. S., *J. Physiol.*, 1912–13, xlv, p. xxii.
Haldane, J. S., *Brit. Med. J.*, 1917, i, 181.
Haldane, J. S., Kellas, A. M., and Kennaway, E. L., *J. Physiol.*, 1919–20, liii, 181.
Haldane, J. S., Meakins, J. C., and Priestley, J. G., *J. Physiol.*, 1918–19, lii, 420.
Hill, L., *J. Physiol.*, 1921, lv, p. xx.
Kline, B. S., and Winternitz, M. C., *J. Exp. Med.*, 1915, xxi, 313.
Krogh, A., *J. Physiol.*, 1918–19, lii, 409.
Lundsgaard, C., *J. Exp. Med.*, 1919, xxx, 147.
Lutz, B. R., and Schneider, E. C., *Am. J. Physiol.*, 1919–20, l, 280.
Meakins, J. C., *Arch. Int. Med.*, 1920, *a*, xxv, 1.
Meakins, J. C., *Brit. Med. J.*, 1920, *b*, i, 324.
Meakins, J. C., *J. Path. and Bact.*, 1921, xxiv, 79.
Meakins, J. C., and Davies, H. W., *J. Path. and Bact.*, 1920, xxiii, 451.
Meltzer, S. J., *J. Am. Med. Assn.*, 1917, lxix, 1150.
Oliver, T. H., and Murphy, D. V., *Lancet*, 1920, i, 432.
Smith, J. L., *J. Physiol.*, 1899, xxiv, 19.
Stadie, W. C., *J. Exp. Med.*, 1919, xxx, 215.
Stadie, W. C., *J. Exp. Med.*, 1922, xxxv, 323.
Stuertz, E., *Z. diätet. u. physik. Therap.*, 1903, vii, 67, 159.
Tunnicliffe, F. W., and Stebbing, G. F., *Lancet*, 1916, ii, 321.
Vernon, H. M., *J. Physiol.*, 1906–07, xxxv, 53.
Verzár, F., *J. Physiol.*, 1912–13, xlv, 39.

AN ORGANISM RESEMBLING BACILLUS ACTINOIDES ISOLATED FROM PNEUMONIC LUNGS OF WHITE RATS.

By F. S. JONES, V. M. D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

PLATE 10.

(Received for publication, October 20, 1921.)

During the past few years several have isolated organisms from rats suffering from pneumonia. Mitchell[1] cultivated from the lungs of rats during an outbreak of pneumonia a granular Gram-positive bacillus. Klein[2] had previously encountered a similar bacillus in two cases of rat pneumonia. This organism he named *Bacillus muris*. *Bacillus muris* grew well on the ordinary media. It produced acid in dextrose broth or dextrose serum water. Cultures injected into the pleural cavity of rats and mice set up a pleuritis and pneumonia with fatal termination. Subcutaneous injection into rats and guinea pigs produced local abscesses. Tunnicliff[3] succeeded in cultivating a delicately growing streptothrix from the lungs of twenty rats. This organism failed to grow on ordinary media but good growths were obtained on media which contained ascitic fluid. The streptothrix was Gram-negative. It was not acid-fast. Branching was observed only in young cultures. It was described as slender, straight or wavy filaments which soon tended to fragment. The culture was pathogenic for rats when injected into the pleural cavity. Recently Hoskins and Stout[4] have obtained *Bacillus bronchisepticus* from the upper respiratory tract and lungs of rats suffering with respiratory disease. From descriptions encountered in the literature one would assume that several types of lung disease exist in the rat.

The writer undertook the study of rat pneumonia and succeeded in obtaining from eleven cases organisms closely resembling those isolated by Theobald Smith[5,6] from the pneumonic lungs of calves.

---

[1] Mitchell, O. W. H., *J. Infect. Dis.*, 1912, x, 17.

[2] Klein, E., *Centr. Bakt.*, *1te Abt.*, *Orig.*, 1903, xxxiii, 488.

[3] Tunnicliff, R., *J. Infect. Dis.*, 1916, xix, 767.

[4] Hoskins, H. P., and Stout, A. L., *J. Lab. and Clin. Med.*, 1919–20, v, 307.

[5] Smith, T., *J. Exp. Med.*, 1918, xxviii, 333.

[6] Smith, T., *J. Exp. Med.*, 1921, xxxiii, 441.

He has named the calf organism *Bacillus actinoides, n. sp.* (Smith, 1918). Certain well defined morphological characters which he described render its recognition a relatively simple matter. His cultures when injected into the tracheæ of calves produced circumscribed necroses of the lung. Subcutaneous injections gave rise to large indurations ending in necrosis.

It must not be assumed that the *Bacillus actinoides*-like cultures were obtained from every rat examined. In a few instances *Bacillus bronchisepticus* was isolated. Cocci of various kinds were frequently found. Streptothrices were observed in a few tubes. In many instances growth could not be obtained.

Cultivation from the lungs of rats presents certain difficulties. Involved lobes are usually shrunken and afford very little material. The disease is a chronic one, offering excellent opportunity for invasion with more rapidly growing bacteria. Experimental work with organisms isolated is equally difficult. Practically all rats which have come under my observation after reaching maturity have spontaneously developed lung disease.

Nothing definite can be said at this time regarding the real relationship of the organism about to be described to certain rat pneumonias. On the other hand, the remarkable similarity which exists between Smith's calf cultures and those I have obtained from the white rat is worthy of emphasis. The pneumonic processes of the rat disease resemble in certain respects those described by Theobald Smith in calves.

Method of Cultivation.

The rat is chloroformed. The skin over the thorax is shaved and disinfected. After the musculature has been exposed it is seared. Portions of the ribs and sternum are removed with sterile instruments. In the main the culture methods described by Smith have been employed. Small pieces from the involved lobes are placed in tubes containing the solid media. The pieces are pushed down the tube and over the surface of the slant and lodged in the liquid at the bottom. The tube is then sealed with sealing wax. Slanted coagulated horse serum to which 0.5 cc. of sterile calf serum water has been added to the condensation fluid has on the whole given the best results. Growth has also been obtained on slanted veal infusion agar to which either calf serum water or defibrinated horse blood has been added to the original condensation fluid. Emphasis is placed on the thorough sealing of the tubes.

Growth Characters.

3 or 4 days after inoculation with bits of lung tissue, tiny scintillating particles may appear within the condensation fluid and calf serum water. As a rule colonies on the slant are not visible to the unaided eye. With a lens, however, very tiny white colonies are observed. The tiny particles gradually become larger and finally appear as irregular gray flocculi suspended in the liquid. In certain cultures a distinct cream-like surface layer in the condensation fluid has been observed in old cultures. The surface colonies increase in size and become round, white, smooth protrusions (Fig. 1). On the agar media the growth is more delicate. Here the condensation fluid becomes slightly turbid and either a very delicate translucent film or discrete colonies appear on the slant. The colonies are very small, usually under 0.5 mm. in diameter. They are round, translucent, and raised. The growth in the fluid at the bottom tends to become viscid. After the organism becomes established on the artificial media it is possible to study its morphological characters in detail. The coagulated serum cultures are in many respects most interesting.

If a 48 hour subculture on coagulated serum be examined with the naked eye little except barely visible scintillating particles in the condensation fluid attracts attention. Hanging drop or stained preparations from the liquid show that the particles are made up of aggregates of fairly long, slender, non-motile rods. They appear to be embedded in clear (unstainable) areas (Fig. 3). Chains of four or five individuals are not infrequent. Preparations from the slant reveal similar organisms. At the end of 4 days the particles have increased in size and appear as tiny gray flocculi. Microscopically they contain many interwoven filaments. A few may protrude from the edges of the mass. The organisms appear granular. About the borders of certain clumps one may encounter a few clear, pear-shaped bodies. Preparations from the slant reveal a few rod-like elements and great numbers of rounded refringent bodies in clumps. When the condensation water is examined after 7 days, the flocculi are larger. They now appear as mulberry masses of pear, slipper, or club shape, or rounded, clear globules. Scattered throughout this substance are small groups of crystals. The structure of the mass is best shown by placing a drop of the liquid on a slide, with gentle pressure the club-like elements can be made to flow. Fig. 2 illustrates the appearance of a well developed flocculus in the condensation water. The details of the edge of a similar flocculus from an older culture show the characteristic clubs and a few crystals (Fig. 4). The resemblance between these club-like masses and preparations of the ray fungus from actinomycotic lesions is striking. Stained preparations add little of value. The capsular substance does not stain with any of the ordinary bacterial stains. As a rule a few very faintly stained filaments and fragments are visible in the center of the flocculus (Fig. 5). Theobald Smith[7] in a recent article has described in detail the formation of the capsular material. The clubs appear first as terminations of the filaments. He points out that the original substance resembles myelin, but that fat granules make their appearance as the culture becomes older and continue to develop at the expense of the myelin-like substance. On the surface of the slant in 7 day cultures the clubs are usually much smaller. Many of the long rods have fragmented so that it is possible to observe masses of very short refringent forms and long filaments. Both types are illustrated in Fig. 6. The shorter forms stain poorly, the long filaments are more deeply colored. Branching has not been observed.

On the blood agar media a much different process is revealed. Here the rods in the young cultures (48 hours) vary considerably in length. Rods from 5 to 10 microns are observed. They are usually straight or slightly curved and often beaded. The ends may be tapered or the bacillus may terminate in a distinct swelling. Constrictions of the body of the organism are not uncommon. Many of these forms have been photographed in Fig. 7. After 5 to 7 days the rod forms have disappeared and hanging drop or wet mount preparations reveal only refringent coccoid elements (Fig. 8). It has been stated that rods and filaments with swollen ends are noted during the first 48 hours of incubation. The swellings become more marked during the 3rd and 4th days. The body of the organism shrinks and finally all that remains is the refringent spore-like mass. These masses when introduced into fresh media will reproduce the characteristic pleomorphic rods which undergo the changes described.

---

[7] Smith, T., *J. Exp. Med.*, 1921, xxxiv, 593.

Although such organisms have been observed in the cultures from eleven cases, it is only with difficulty that they can be maintained. In several instances *Bacillus actinoides* was associated with a delicately growing streptococcus. It was possible to carry along these mixed cultures in serum media for several generations, ultimately only the streptococci survived. These mixed cultures made the microscopic study exceedingly difficult. It was only after a few pure cultures of the rods were obtained that the nature of the growth process became clear. Thus far it has not been possible to transfer cultures from the coagulated serum media to blood agar or calf serum water agar. Attempts to transfer from the agar to serum media have also failed.

The organism when grown on agar stains easily in young cultures with methylene blue or carbolfuchsin. It is Gram-negative. Preparations from the serum media are more difficult to stain.

The pathogenic properties of certain strains have been tested in a few instances. White rats injected with cultures beneath the skin suffered no ill effects. Several experiments were made in which the culture was introduced into the trachea of rats. Unfortunately the controls developed a spontaneous pneumonia so that nothing definite could be determined. A calf injected beneath the skin of the neck with a typical rat culture developed only a mild local reaction.

SUMMARY.

An organism obtained from the pneumonic lungs of eleven white rats is described. It has been maintained in pure culture in a number of instances. In young cultures the organism appears as a long slender bacillus. In older cultures on coagulated serum media characteristic club-like capsular material is formed. On blood agar characteristic swellings appear at one or both ends of the rod. These become more refringent, and the body of the organism begins to shrink. Finally only rounded spore-like refringent bodies are found. The organism is Gram-negative.

EXPLANATION OF PLATE 10.

Fig. 1. A culture, 3 months old, from the ventral lobe of Rat 1, Experiment 5, on coagulated horse serum. The surface growth is characteristic, a few larger flocculi are visible suspended in the condensation water. Many have sunken to the bottom. Natural size.

Fig. 2. A large flocculus and several smaller ones from the condensation fluid of a 7 day serum culture. × 25.

Fig. 3. Stained preparation from the condensation fluid of a 48 hour coagulated horse serum culture, Rat 53. Note the aggregates of rods in the clear spaces. Stain is dilute carbolfuchsin. × 1,000.

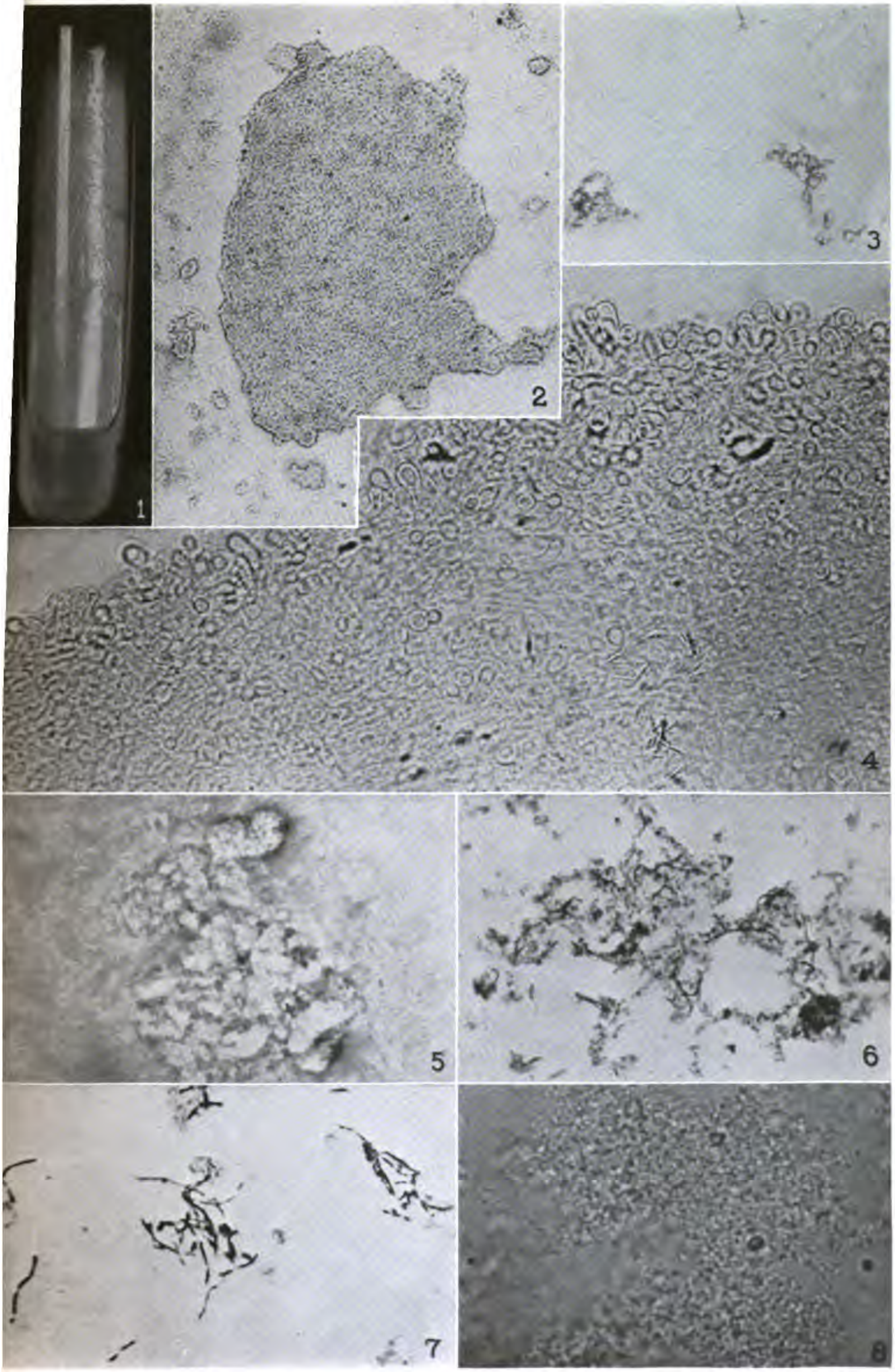
Fig. 4. The edge of a flocculus similar to that shown in Fig. 2. The preparation is from the liquid of a 2 weeks old culture on coagulated serum. The clubs in several planes appear as protruding pear-shaped, rounded, or oval, clear bodies. A few needle-like crystals are present. × 550.

Fig. 5. A flocculus from the condensation fluid of a 7 day serum culture stained with methylene blue for 12 hours. A few faintly stained filaments and fine granules are visible. The capsular substance is unstained. × 1,000.

Fig. 6. Stained preparation from the slant of a 7 day culture on coagulated serum. Note the long filamentous forms which stain well and the fragments which stain only faintly. One rod appears to have a branch leaving the main filament at an angle of about 45°. The microscope reveals that these organisms are entirely separate. The stain is dilute carbolfuchsin. × 1,000.

Fig. 7. A stained preparation from the slant surface and condensation fluid of a 48 hour blood agar culture, Rat 42. Various forms are clearly illustrated. × 1,000.

Fig. 8. An unstained wet mount from the lower portion of the slant and condensation fluid of a 12 day blood agar culture, Rat 42, showing the refractile spore-like bodies. × 650.



(Jones: Organism from pneumonic lungs of rats.)

A THREE MONTHS OLD STRAIN OF EPITHELIUM.

BY ALBERT FISCHER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 11 TO 14.

(Received for publication, October 20, 1921.)

The purpose of the experiments described in this paper was to obtain a pure strain of epithelium and to keep it permanently *in vitro*, as has been done with connective tissue. Epithelium taken from chickens, dogs, human beings, etc., has already been grown *in vitro* for a short period of time and its characteristic growth described.[1] Ruth,[2] Oppel,[3] Holmes,[4] and Uhlenhuth[5] have cultivated epithelium from cold-blooded animals. Carrel[6] attempted several times to obtain a permanent strain of epithelium from the skin of chick embryos, but after 2 or 3 weeks the cultures were invaded by fibroblasts and the epithelial cells progressively disappeared. The transformation of the cultures of epithelium into cultures of connective tissue was probably not caused by dedifferentiation of epithelial cells into fibroblasts, as has been stated by Champy,[7] but by contamination of the cultures by fibroblasts. The fibroblastic contamination was due to the difficulty of obtaining for culture epithelium completely free from connective tissue cells. Ebeling[8] succeeded in keeping pure cultures of corneal epithelium which spontaneously ceased to grow after a few weeks. Although the cultivation of epithelium appeared to be more difficult than that of connective tissue, it seemed probable that if epithelial cells could be obtained free from connective tissue cells, they could be kept in pure culture indefinitely. The strain of epithelium described in this article was isolated from chick embryo eyes. Fragments of tissues were taken from different parts of the eye, tapetum layer, cornea, and lens, in the hope of obtaining pure epithelium. Only a few cultures from the lens produced pure epithelium, and by repeating the explantation of different tissues from the eye, it appeared that only a certain kind of supposed lens cultures produced a pure outgrowth of epithelium, namely, the peripheral portion which had a little brim of iris epithelium attached. The lens tissue itself did not grow at all, but the little brim of iris which sticks to the lens when it is enucleated grew out apparently as pure epithelium. After 3 months cultivation *in vitro* it still looks as pure as when it was observed on the 1st day. Now and then a few elongated, spindle-shaped cells resemble fibroblasts, but under high magnification they are easily recognized as epithelial cells on account of their similarity to the other cells in the culture, the pigment which the iris epithelium contains, and their characteristic way of growing in close contact with the neighboring cells.

---

[1] Carrel, A., and Burrows, M. T., *J. Am. Med. Assn.*, 1910, lv, 1379; *J. Exp. Med.*, 1911, xiii, 416.

[2] Ruth, E. S., *J. Exp. Med.*, 1911, xiii, 422.

[3] Oppel, A., *Anat. Anz.*, 1913–14, xlv, 173.

[4] Holmes, S. J., *Univ. California Pub. Zool.*, 1913, xi, 155.

[5] Uhlenhuth, E., *J. Exp. Med.*, 1914, xx, 614.

[6] Carrel, A., unpublished experiments.

[7] Champy, C., *Bibliog. Anat.*, 1913, xxxiii, 184.

[8] Ebeling, A. H., unpublished experiments.

I.

EXPERIMENTAL.

1. Preparation of the Tissue.—Epithelial tissue for cultures is obtained from the eye by taking out the lens with a cataract knife. In most cases a very thin black brim of the iris adheres spontaneously to the peripheral part of the lens. The lens may be cut in three or four parts and placed in the ordinary plasma-tissue juice culture medium. The lens itself does not grow, but sometimes the outgrowth of epithelium from the iris can be observed after 48 hours, although frequently not until after several passages. If any fibroblasts grow out in the same culture, which will be noticed readily, there is little hope of obtaining pure epithelium from it. If parts of the iris are allowed intentionally to remain on the lens, the growth will be largely composed of fibroblasts. The extirpation is carried out as if only the lens is to be removed, and only that part of the iris which spontaneously remains is suitable for obtaining a pure epithelial culture.

2. Nature of the Medium.—The fact that the epithelial cells liquefied the plasma clot much more extensively than fibroblasts suggested that if the cells were cultivated under conditions in which a kind of liquefaction has already taken place, a more extensive and uniform growth would result. An attempt was then made to place the tissue on the free surface of the already coagulated plasma (one volume of plasma and one volume of embryonic juice), and to cover it with a small drop of embryonic tissue juice. This method of cultivation appeared to be the most satisfactory. As a rule, the growth took place in one delicate, continuous layer on the surface of the clot.

3. Preparation of the Cultures.—Equal volumes of plasma and embryonic tissue juice are mixed on the cover-slip and allowed to coagulate. After coagulation commences, which can easily be ascertained with the point of the knife, the tissue is placed on the free surface of the nascent clot. If this precaution is not taken, the tissue will float in the tissue juice which is afterward added, and no growth will occur. It is important to place the tissue on the clot at the proper moment; if too early, the tissue will be embedded, and if too late, it will not adhere. Fixation is the desired object. After the fragment has been properly placed on the clot, a small drop of embryonic juice is spread evenly, in a thin layer over the tissue and beyond its margin, to afford a moist surface for proliferation. When transferring the culture, the excess fluid is removed by means of a piece of sterile filter paper, and the culture cut in the usual way. Because of the retraction of the tissue, it is most important to extirpate the tissue in such a way that no part of the plasma clot remains in the periphery of the excised culture. Otherwise, the cells will be embedded in old clot and further growth will be prevented.

It is advisable to embed the cells in the medium during the first few passages. If connective tissue cells should be present, they are much more easily distinguished, because of their characteristic appearance when they grow in the middle of the clot. When no fibroblasts can be detected, the cultures are allowed to grow on the free surface of the clot, covered only by a small drop of tissue juice.

II.

RESULTS.

The outgrowth of new cells appears in a fine mosaic structure. The size of the epithelial layer of conglomerated cells depends very much on the consistency of the media. When cultivated on the free surface of the plasma clot, the new growth appears as a continuous sheet of cells in pavement formation (Fig. 1). When cultivated in the middle of the clot, smaller and larger peninsulas of cells grow out, and sometimes a few single cells.

For some while there has been a discussion as to the reason for the disappearance of epithelium when cultivated *in vitro*. This fact has been brought out in connection with an assumption by Champy[7] that a dedifferentiation of the epithelial cells to the type of fibroblasts takes place *in vitro*. This does not seem to be correct, and is more an apparent phenomenon. Several investigators, among them Uhlenhuth,[9] have shown that the shape of the cells cultivated *in vitro* depends upon the structure and consistency of the culture medium; in other words, on purely mechanical facts. By taking the precaution of cultivation under the same mechanical conditions, namely on a moist surface of plasma clot, as herein described, the cultures present as typical epithelial characteristics after 3 months cultivation as they did on the 1st day. Whenever it is desired, the epithelial cultures can be embedded in the solid plasma clot and the supposed dedifferentiated epithelial cells can be reconstructed; and repeated cultivation on the surface brings the fusiform cells back to the polygonal type of epithelium. It is purely a matter of mechanical conditions. In the periphery of ordinary, well grown epithelial cultures, made on the surface of the clot, some more or less elongated, spindle-shaped cells appear. These are found mostly when the outlines of the growth reach the outlines of the moist surface; *i.e.*, the border of the area which is covered by the tissue juice. Then they embed themselves in the solid clot and become elongated because of the dense medium. This same phenomenon has been described by Uhlenhuth[5] in his experiment on epithelial cultures from frog skin. He seems to have found just the reverse of the observation stated above; namely, that the elongated, fusiform, epithelial cells grow out only in the very "soft medium," and in compact sheets when cultivated in a "firm medium." This may be explained in the following way: when the culture medium is made as soft as in Uhlenhuth's[9] experiments, but not quite fluid, the fibrin fibrillæ are so sparsely present that the few cells which grow out stick to them as the only support, and become spindle-shaped for that reason. The same phenomenon occurs when tissue is cultivated in a fluid medium in which cotton threads[10] or a spider net (Harrison[11]) is used for framework. In some cases, when cultures were made in the firm clot, it may be that the tissue cells found their support dense and homogeneous. In others, the transplanted fragment of tissue may have been more or less near the surface of the clot and, for that reason, the cells may have grown out on the surface in continuous layers.

---

[9] Uhlenhuth, E , *J*. *Exp*. *Med*., 1915, xxii, 76.

The epithelial strain now under cultivation is very easily multiplied. The strain took its origin from five cultures, and in the elapsed time they have been increased to between 50 and 60, though the speed of the growth seems to be slower than that of fibroblasts. The ability to proliferate seems also to have increased recently; whether this is due to an improved technique in making the cultures or whether the cells have become better adapted to the life *in vitro* is difficult to say at present. It is noteworthy that the cultures made from fresh tissue are often continued for several passages before the epithelium begins to grow. A similar stage of latency has been observed by Uhlenhuth[9] in epithelial cultures.

The epithelial cells in cultures cause a liquefaction of the plasma clot to a higher extent than do those of connective tissue cells, and the epithelial tissue itself when excised from the culture has a peculiar mucous character and retracts easily, as does a drop of mucus. The most characteristic feature about the growth of epithelium is that the cells keep close together and grow in pavement formation. Numerous mitoses are found in the cultures and different stages of amitotic cell division (Figs. 2, 3, and 5). Most of the investigators who have cultivated epithelial cells *in vitro* state that they did not observe any mitosis. After cell division, the new cells do not seem to move very far from the point where the division took place. The photographs show such an ability to form a continuous layer (Figs. 1 to 4). Very often in looking at the cultures, one is reminded of a cross-section of epithelial glandular tissue; that is, not only do the cells grow in pavement formation, but they seem to have a tendency to arrange themselves in a structure resembling the acini of glandular tissue (Figs. 2, 4, and 6). No dedifferentiation such as that suggested by Champy[7] has taken place after 3 months cultivation *in vitro*. The appearance of the cells at the present time is identical with that observed after the first passage. The rate of proliferation is increasing. Every culture doubles in size after 3 or 4 days.

---

[10] Fischer, A., unpublished experiments.

[11] Harrison, R. G., *J. Exp. Zool.*, 1914, xvii, 521.

III.

CONCLUSIONS.

1. A strain of pure epithelial cells was obtained from the part of the iris which adheres to the extirpated lens.

2. The optimum condition under which epithelial cells grow *in vitro* is on the free surface of the plasma clot under a film of embryonic tissue juice.

3. The epithelial cells did not dedifferentiate. Although the strain is 3 months old, they grow as a pavement membrane and have kept their epithelial characteristics.

EXPLANATION OF PLATES.

PLATE 11.

FIG. 1. Photograph of a living culture of epithelium 6 weeks old. Shows the characteristic growth for epithelial cells. × about 135.

FIG. 2. Stained culture of epithelial cells, 6 weeks old. Shows the epithelial pavement formation; mitosis may be seen. A few proliferation centers may be noticed. × about 660.

PLATE 12.

FIG. 3. Stained culture of epithelial cells, 6 weeks old. To the left may be seen the beginning of an amitotic cell division. × 1,100.

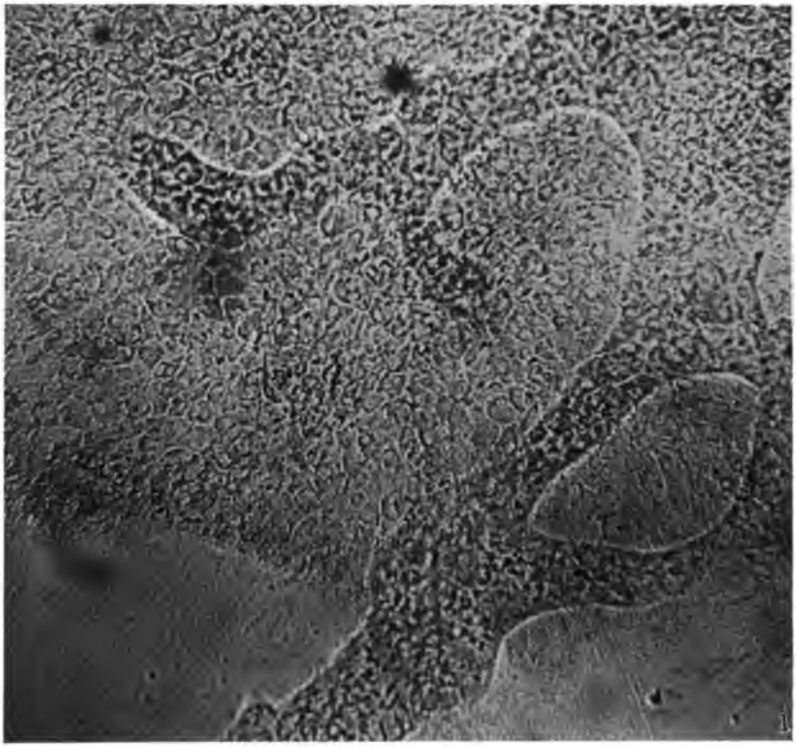
FIG. 4. Stained culture of epithelial cells 2 months old. × 1,425.

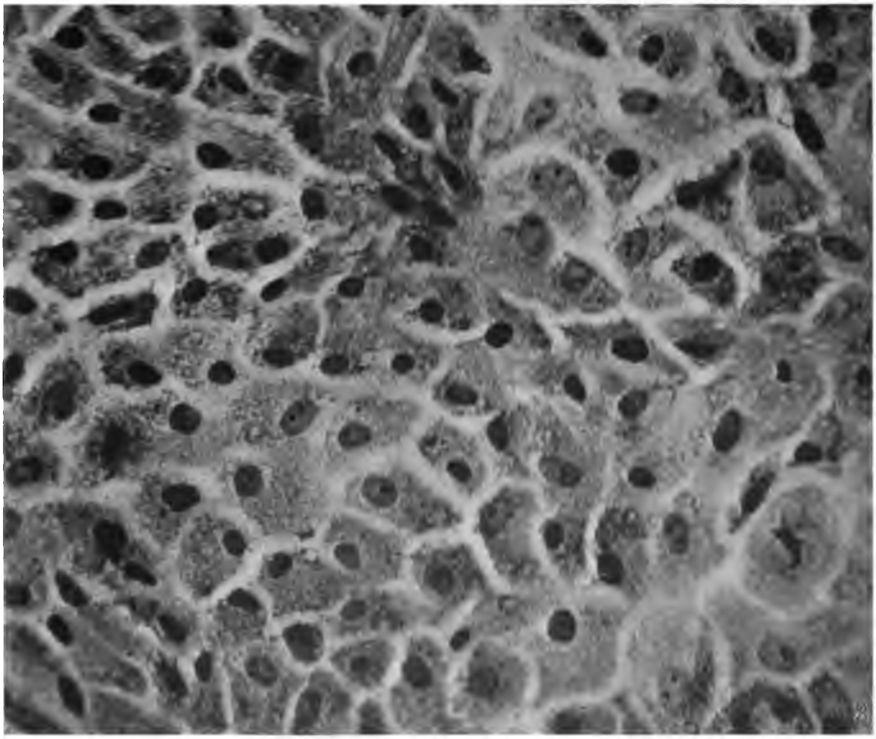
PLATE 13.

FIG. 5. Different stages of mitosis of epithelial cells in cultures.

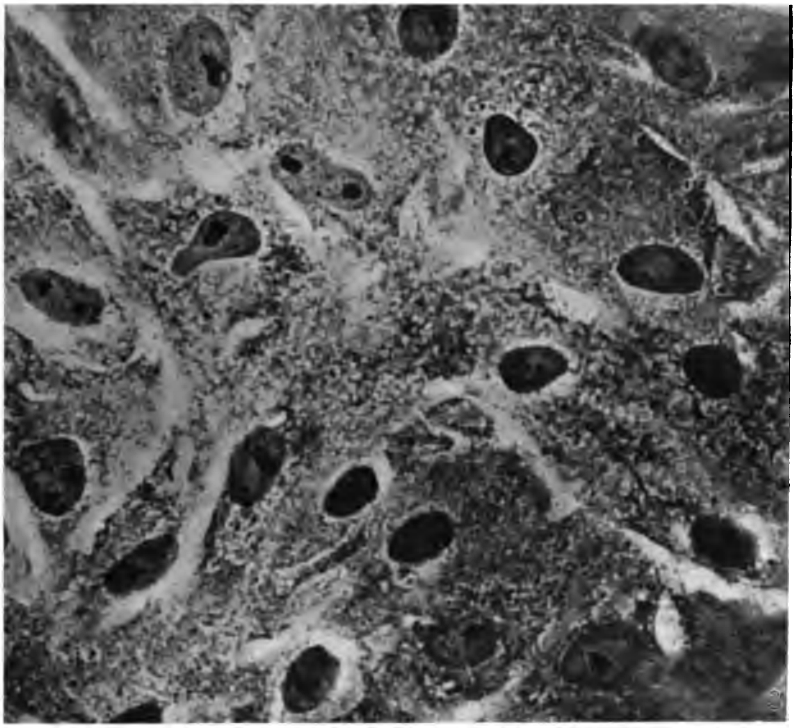
PLATE 14.

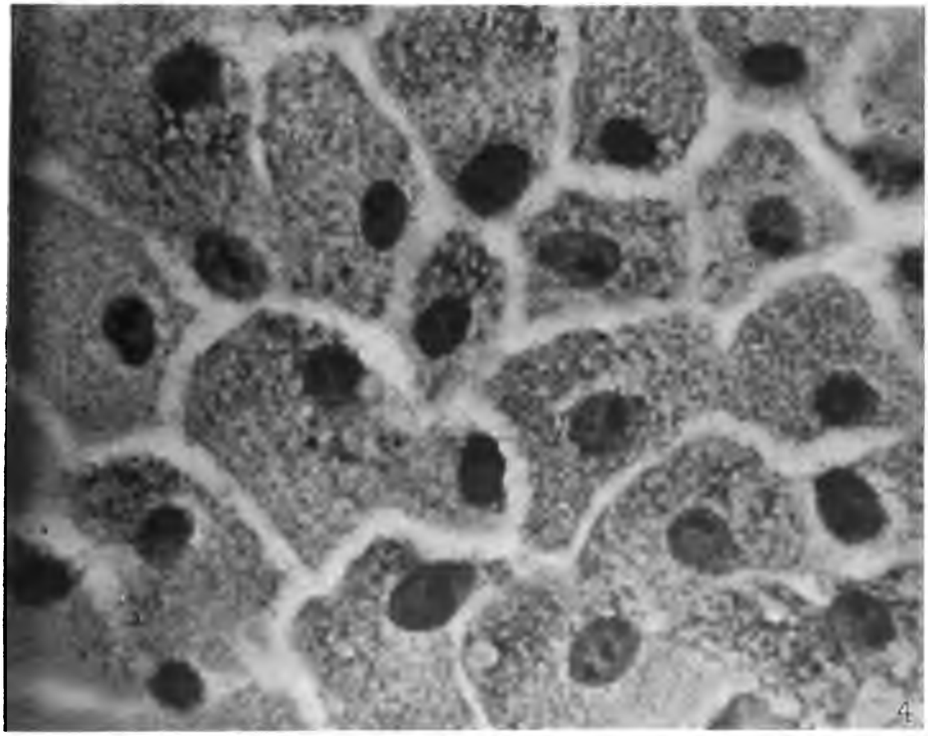
FIG. 6. Typical cell arrangement in epithelial cultures. × 700.



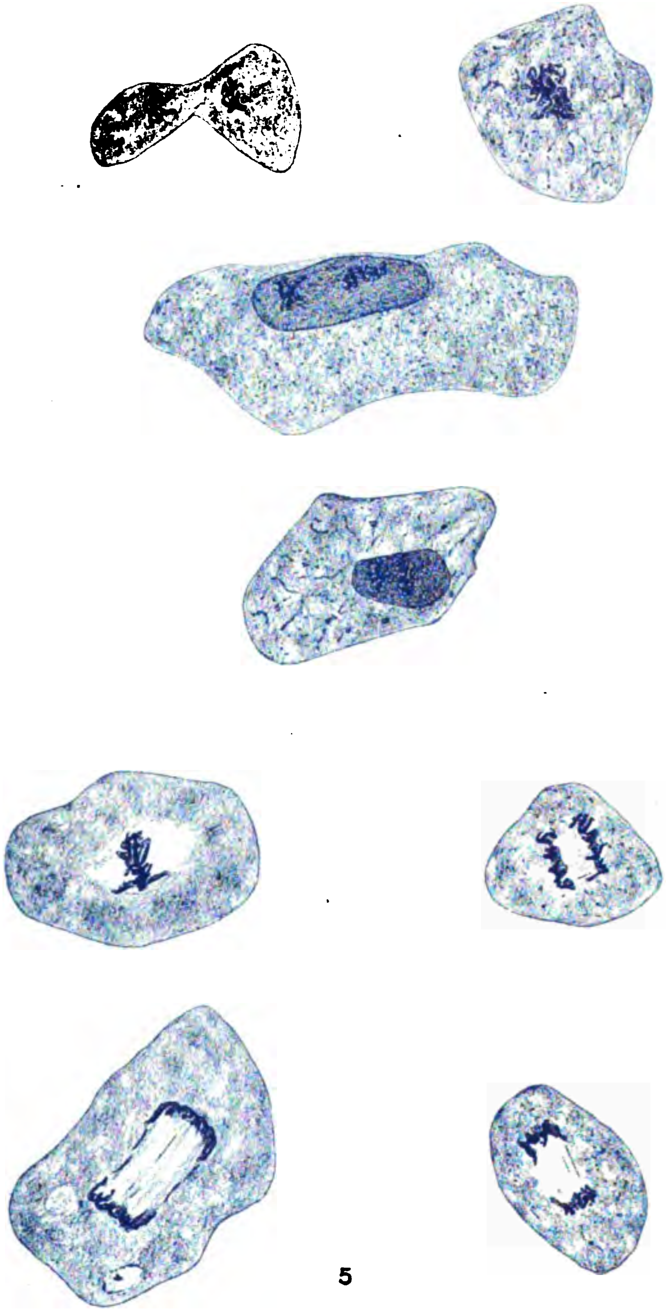


(Fischer: A strain of epithelium.)

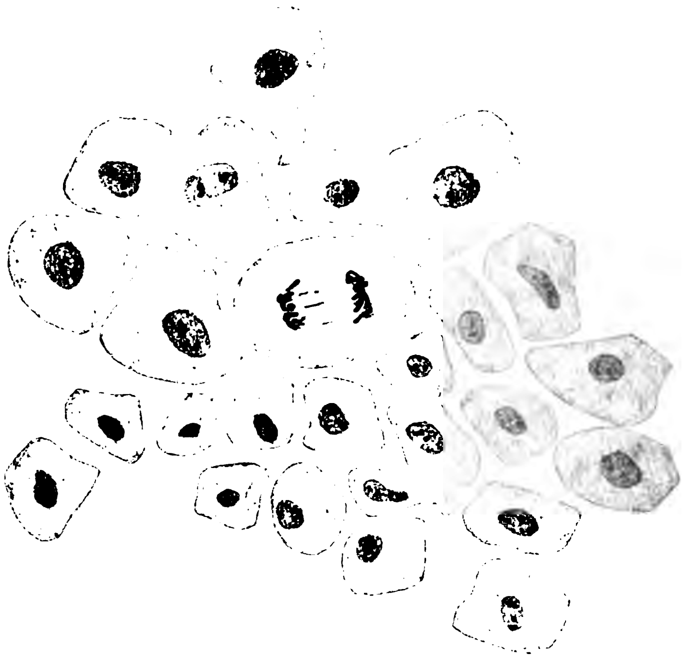


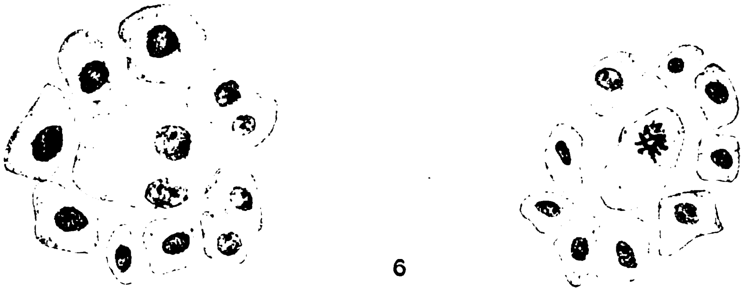


(Fischer: A strain of epithelium.)



(Fischer: A strain of epithelium.)





6

(Fischer: A strain of epithelium.)

STUDIES ON X-RAY EFFECTS.

IX. THE ACTION OF SERUM FROM X-RAYED ANIMALS ON LYMPHOID CELLS IN VITRO.

BY JAMES B. MURPHY, M.D., J. HENG LIU, M.D., AND ERNEST STURM.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 15.

(Received for publication, October 17, 1921.)

In the course of an investigation on the biological effects of x-rays it was noted that while larger doses of this agent destroy lymphoid tissue, very small exposures, after causing a slight amount of destruction, will bring about an actual stimulation of this tissue.[1] The mechanism of the stimulation phenomenon is of considerable interest owing, among other things, to the relation of the lymphoid tissue to cancer resistance. The most satisfactory stimulation has been obtained with x-rays of comparatively long wave-lengths and, therefore, of low penetrating power. In fact, the best results have followed exposure to the rays from a specially constructed tube with a window which permits the emission of a larger proportion of the soft rays than are given off by the standard tubes; and this tube is operated with a spark-gap of ½ inch.[2] Of the very small dose used here, approximately 57 per cent is absorbed by the first ¼ cm. of tissue, and over 92 per cent before the rays have penetrated to the depth of ½ cm., while at a depth of 1½ cm. only 0.56 per cent of the rays remains. It seems extremely doubtful, therefore, whether these rays penetrate to the deeper lymphoid organs in sufficient strength to bring about any change; yet these organs show as much evidence of stimulation or destruction as do the organs which are superficial enough to be directly acted upon by the rays.

---

[1] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800. Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75. Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83. Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1920, xxxi, 13.

[2] Nakahara, W., and Murphy, Jas. B., *J. Exp. Med.*, 1922, xxxv (in press).

This observation has led to a consideration of the possibility of the spleen and lymph gland changes being secondary to some alteration in the circulating blood or other tissues, brought about by the action of the x-rays. The point is one that has already been the subject of several investigations. Linser and Helber's[3] experiments led them to conclude that the serum from x-rayed animals contained a leucotoxin which on injection into other animals produces destruction of the circulating leucocytes. The leucotoxin was destroyed by heating at 55–60°C. The toxin, according to these authors, is transmitted from mother to fetus through the placenta. Capps and Smith[4] reported similar findings, with the serum from successfully treated leucemia patients, and state that the serum from an x-ray-treated case of leucemia when injected into an untreated case causes a definite fall in the number of white blood cells. Curschmann and Gaupp[5] also state that serum from x-ray-treated cases of leucemia, in a dilution of 1:100, causes rapid destruction of leucocytes *in vitro*.

Later Klieneberger and Zoeppritz[6] failed to confirm any of the above experiments, which is true also of other observers[7] with reference to the so called leucotoxin in the serum of x-ray-treated individuals.

With the evidence at hand indicating the indirect action of the x-rays on the lymphoid tissue, it seemed of interest to reopen the question and to determine whether or not the serum of x-rayed animals has any effect on lymphoid cells *in vitro*.

The Effect of Serum from X-Rayed Animals on Lymphoid Cells in Vitro.

A number of healthy young rats were exposed to a dose of x-rays governed by the following factors: spark-gap 2½ inches; milliamperes 10; distance 12 inches; time 14 minutes. Immediately following this treatment the animals were anesthetized and exsanguinated by aspiration of the heart. The blood was placed in a test-tube, and after clotting it was centrifuged. The serum was then drawn off and again centrifuged at high speed to remove any remaining cells or fibrin. Serum was collected in the same manner from a like number of normal rats. After the blood had been drawn from the normal rats, the thymus and mesenteric lymph glands were removed under aseptic conditions. The glands were freed from adherent tissue and were then divided as nearly as possible into equal parts so that two lots were made, each having half of the thymus and half of the mass of mesenteric lymph glands from a number of different rats. One of the portions was then mixed with the serum from normal rats and the other with serum from the x-rayed rats and each was then ground thoroughly in a mortar, and the resulting suspensions were passed through filter paper under suction to remove the fibrous tissue and cell clumps. Counts were made of the two filtrates to determine the number of cells present, and then enough of the two sera was added to reduce the counts to between 10,000 and 20,000 cells per c. mm. Another count was made on the suspensions after they had been well shaken to standardize the suspension. The tubes were tightly plugged and placed in a water bath at 37°C. for 2 hours. They were then removed, well shaken, and counted, and again after 4 hours in the water bath this procedure was repeated. Films were made at the time of each count and stained with Wright's blood stain. The enumeration in each case was made by two individuals on different samples of the suspension and when there was a divergence, the mixtures were reshaken and the counting was repeated. Many counts were checked up with the high power lens of the microscope so as to make sure that fragments and debris were not included.

---

[3] Linser, P., and Helber, E., *Deutsch. Arch. klin. Med.*, 1905, lxxxiii, 479.

[4] Capps, J. A., and Smith, J. F., *J. Exp. Med.*, 1907, ix, 51.

[5] Curschmann, H., and Gaupp, O., *Münch. med. Woch.*, 1905, lii, 2409.

[6] Klieneberger, C., and Zoeppritz, H., *Münch. med. Woch.*, 1906, liii, 850.

[7] Melchner, R., and Wolff, W., *Berl. klin. Woch.*, 1906, xliii, 746.

Table I gives the tabulated results of fourteen such experiments in which Serum A is from normal animals and Serum B from x-rayed animals.

The average of these fourteen experiments (Text-fig. 1) shows that the cells suspended in normal serum decreased by over 3,000 during the first 2 hours and by another thousand by the end of the 4 hour period. The cells in the serum from x-rayed animals increased by over 3,000 cells in the first 2 hours and showed only a slight drop be-

TABLE I.

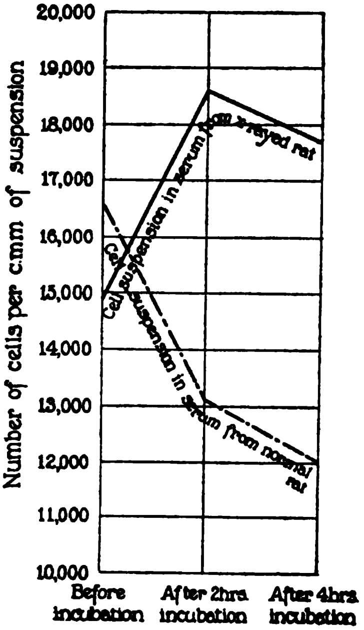
Time.	No. of cells in suspension.													
	Experiment 1.		Experiment 2.		Experiment 3.		Experiment 4.		Experiment 5.		Experiment 6.		Experiment 7.	
	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.
Before incubation...............	18,840	18,250	15,280	17,260	13,900	15,000	23,700	14,500	20,200	12,160	8,180	9,925	16,920	15,500
After 2 hrs. incubation..........	17,340	24,880	15,400	23,550	13,460	20,460	13,080	17,250	17,500	16,000	6,500	11,890	12,200	17,500
" 4 " "	16,960	27,600	13,190	21,100	13,920	18,750	10,080	15,500	16,900	12,500	5,700	9,590	13,000	19,150
" 24 " "	10,050	24,670												

Time.	No. of cells in suspension.													
	Experiment 8.		Experiment 9.		Experiment 10.		Experiment 11.		Experiment 12.		Experiment 13.		Experiment 14.	
	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.
Before incubation...............	19,500	18,500	17,240	17,600	19,100	14,800	17,500	15,040	14,880	15,540	19,040	11,820	9,850	13,800
After 2 hrs. incubation.........	17,100	21,300	13,000	23,200	13,400	15,900	14,450	19,900	11,440	17,160	13,500	15,640	6,300	15,760
" 4 " "	13,240	24,700	15,000	21,550	11,000	14,000	13,300	21,400	8,350	13,830	12,200	13,520	5,300	14,850

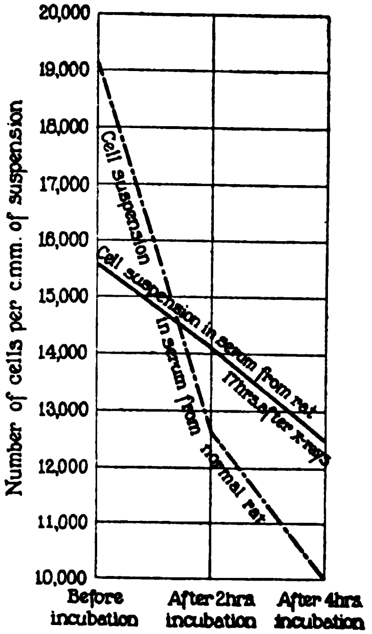
Serum A is from normal rats, Serum B from rats immediately after a dose of x-rays.

tween the 2 and 4 hour periods. At the end of the period of observation the counts showed the suspensions still had some 3,000 cells more per c. mm. than the original suspension.

Examination of a large number of stained films made from these suspensions at the 2 hour period showed among the cells suspended in serum from x-rayed animals a fairly large number of mitotic figures (Fig. 1). The average was a little less than one mitosis to a thin film,



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Graphic representation of the average of Experiments 1 to 14.
TEXT-FIG. 2. Graphic representation of the average of Experiments 15 to 17.

and occasionally three or more were found in a film. In only one instance was a dividing cell found in the normal serum suspension. The amount of disintegration of the cells, judged by the number of degenerated forms found in the smears, is just as rapid in the serum from x-rayed animals as in that from normal animals. Apparently, therefore, the proliferation of the cells in contact with serum from x-rayed animals is sufficient to replace not only the disintegrated cells but also actually to increase the total number. A large number of films prepared from the suspensions before incubation failed to show any mitotic figures, thus ruling out the question of the dividing cells being carried over in any appreciable numbers from the glands.

An unsuccessful attempt was made to extend the above observations to rabbits, but the fragility of the lymphoid cells was such that by the end of the 2 hour period no accurate counts could be made. The cells of guinea pigs showed less tendency to disintegrate and a small increase in the number of cells suspended in serum from an x-rayed animal was noted. However, the rate of disintegration was too rapid to obtain definite or consistent results. Finally, rat lymphoid cells suspended in serum from rabbits were destroyed so rapidly as to make it impossible to secure accurate counts.

The Duration of the Stimulative Quality of Serum from X-Rayed Animals.

In order to test the length of endurance of the stimulative effect of the serum from x-rayed rats, the above experiments were repeated, except that in this series the blood was taken 17 hours after the x-ray treatment was given. The results of these observations are given in Table II.

TABLE II.

Time.	No. of cells in suspension.					
	Experiment 15.		Experiment 16.		Experiment 17.	
	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.
Before incubation............	23,700	15,500	14,880	17,480	19,040	14,100
After 2 hrs. incubation.......	14,080	14,350	11,440	15,240	13,500	12,700
" 4 " "	10,080	14,750	8,350	13,250	12,200	12,500

Serum A is from normal rats, Serum B from rats 17 hours after a dose of x-rays.

It will be seen that active stimulative effect of the serum from x-rayed rats is lost by 17 hours after the treatment (Text-fig. 2), but it may be noted that the rate of disintegration is retarded somewhat in the serum from the x-rayed animals. It is not clear whether the retarding action represents an actual slowing down of the disintegration rate or whether there is enough stimulation substance remaining to bring about a less rapid fall in the count by cell multiplication. The finding of one mitotic figure in the comparatively small number of preparations studied suggests the latter possibility.

The Effect of Serum X-Rayed in Vitro on Lymphoid Cells.

A quantity of serum was prepared from normal rats in the same manner as in the preceding experiments. Half of this was exposed directly to x-rays in the same amount as that given to the animals in the previous experiments.

The cell suspensions were prepared in the same manner as described above with the normal and x-rayed serum. The results of these experiments are given in Table III.

TABLE III.

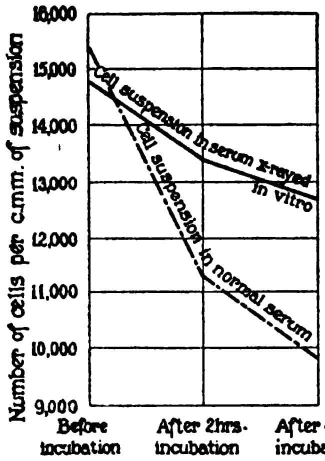
Time.	No. of cells in suspension.					
	Experiment 18.		Experiment 19.		Experiment 20.	
	Serum A.	Serum B.	Serum A.	Serum B.	Serum A.	Serum B.
Before incubation	19,100	16,000	17,500	15,200	9,850	13,360
After 2 hrs. incubation	13,400	14,100	14,450	14,350	6,300	11,960
" 4 " "	11,000	14,100	13,300	13,300	5,300	10,900

Serum A is from normal rats, Serum B is normal rat serum exposed to x-rays *in vitro.*

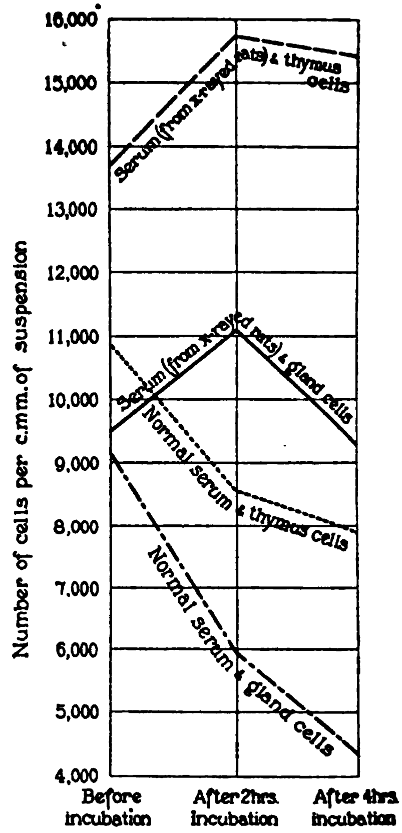
Thus the serum x-rayed *in vitro* proved to be devoid of stimulative effect on the suspended lymphocytes, but as in the preceding experiment there was a retardation of fall in the cell count (Text-fig. 3).

Variations in the Response of Lymphoid Cells to Stimulative Effects.

In all the experiments described above, the cell suspensions were prepared from the thymus and mesenteric lymph glands usually of five rats. Each mass of glands was divided into two parts so that the final suspensions contained about equal amounts of the tissue from each animal used in the experiment. It will be noted in the figures given that there was considerable variation in the amount of stimulation in the various experiments, although the dose of x-rays was the same throughout. Hence a test was made to ascertain whether the cells of the thymus and the lymph gland shared equally in the stimulation and also whether there was striking individual variation in the degree of response between cells of different animals.



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. Graphic representation of the average of Experiments 18 to 20.
TEXT-FIG. 4. Graphic representation of the average of Experiments 21 to 25.

Serum from normal and x-rayed rats was prepared according to the method described in the first series of experiments. The thymus from two rats and the lymph glands from the same two animals were prepared separately so as to yield a suspension of thymus cells in serum from x-rayed rats, and a suspension of thymus cells from the same

TABLE IV.

Time.	No. of cells in suspension.															
	Experiment 21.				Experiment 22.				Experiment 23.				Experiment 24.			
	Serum A + lymph glands.	Serum A + thymus.	Serum B + lymph glands.	Serum B + thymus.	Serum A + lymph glands.	Serum A + thymus.	Serum B + lymph glands.	Serum B + thymus.	Serum A + lymph glands.	Serum A + thymus.	Serum B + lymph glands.	Serum B + thymus.	Serum A + lymph glands.	Serum A + thymus.	Serum B + lymph glands.	Serum B + thymus.
Before incubation....	11,450	4,000	9,900	9,500	9,000	11,400	18,450	14,350	8,450	14,250	5,200	14,250	7,850	14,100	4,400	16,500
After 2 hrs. incubation..............	7,600	2,550	10,450	12,030	6,350	9,160	22,500	17,700	5,970	10,150	6,970	12,800	4,000	12,400	4,300	20,600
After 4 hrs. incubation.............	6,500	2,150	4,850	13,350	3,800	8,400	22,000	17,800	3,850	9,650	6,500	12,300	3,300	11,500	3,700	18,150

Serum A is from normal rats, Serum B from rats immediately after a dose of x-rays.

animals in normal serum. Two like suspensions of the lymph glands from the same animals in the two sera were prepared for comparison. Table IV shows the result of four such experiments.

Another like experiment was carried out, to test the response of glands from different groups of rats to the same serum from x-rayed animals.

The figures for these experiments are given in Table V.

TABLE V.

Time.	No. of cells in suspension.					
	Experiment 25.					
	Serum A + lymph glands.	Serum A + thymus.	Serum B + thymus.	Serum B + lymph glands (1).	Serum B + lymph glands (2).	Serum B + lymph glands (3).
Before incubation............	16,970	14,000	10,200	17,800	2,800	3,750
After 2 hrs. incubation.......	11,600	9,100	11,840	18,800	4,360	4,150
" 4 " "	11,070	8,450	12,500	16,000	3,950	4,200

Serum A is from normal rats, Serum B from rats immediately after a dose of x-rays.

It is obvious from these experiments that the thymus and lymph gland cells are affected about equally by the serum from x-rayed animals (Text-fig. 4), although the thymus cells from some animals respond more readily than the lymph gland cells from the same animals, while in others the opposite is true.

There is also considerable variability in the stimulative power of the same serum on the lymphoid cells of different individuals.

The Effect of the Serum from Animals after a Very Large Dose of X-Rays on Lymphoid Cells.

In further experiments, an attempt has been made to determine whether there is a destructive action on the lymphoid cells of serum from animals after a very large dose of x-rays. Rats were exposed for an hour to a dose of x-rays, otherwise governed by the same factors as in the preceding experiments, and the effect of the serum of these animals was tested on lymphoid cell suspension. There was no evidence of a stimulative effect, nor was there any more rapid disintegration of the cells than was observed to take place in the normal serum.

DISCUSSION.

The experiments reported here fail to show any evidence of the presence of a so called lymphotoxin in the serum of x-rayed animals, even after an exposure so large as to cause almost complete destruction of the lymphoid tissue of the living animals. It is true, however, that these experiments are not an exact repetition of the earlier work along this line, but it seems probable that if any such leucotoxic substance was present in the serum of x-rayed animals, some indication would have appeared among our results. It is difficult to conceive of a lymphotoxin of such power as to be effective in dilutions of 1:100 resulting from a comparatively small dose of x-rays given to a leucemic patient. It is much more difficult to judge the reported results of the injection of serum from an x-rayed individual into animals, for there is the complicating effect of the foreign protein reaction to be taken into account, as well as the instability of the blood counts of the rabbit and guinea pig, the animals used for these tests. In regard to the latter point our experience has been that it is necessary to resort to extreme measures of precaution in order to get a fairly stable blood picture in such animals.

The source and character of the stimulus for lymphoid cells contained in the serum from x-rayed animals are questions about which there is as yet little to be said. The fact that this stimulative quality is not possessed by serum x-rayed *in vitro* suggests that the change is not a simple one in the serum itself. Furthermore, it is known that the stimulation of lymphocytes induced by x-rays *in vivo* is always preceded by a certain amount of destruction of lymphoid cells, a fact suggesting the possibility of the stimulating substance being of the nature of a disintegration product of lymphoid cells.

There is ample proof, both from the cell counts and the presence of mitotic figures, that multiplication actually takes place in cells in a fluid medium, although it has generally been supposed that a matrix of some kind is a necessity for growth. No other explanation of the results described is apparent than that cells are capable of being stimulated to active multiplication in a fluid medium, and that such a stimulative agent is present in the serum of x-rayed animals. How great a part this agent plays in the stimulation observed to take place *in vivo* after a small dose of x-rays is still to be determined.

SUMMARY.

Lymphoid cells, prepared from the thymus and lymph glands of rats, when suspended in the serum of x-rayed rats and incubated for 2 hours, increase in number from 15 to 30 per cent, and mitotic figures are found among these cells in fairly large numbers. A like suspension of cells in normal serum undergoes rapid disintegration and in only one instance among a large number of films examined was a mitotic figure found.

The stimulative effect of the serum from x-rayed rats endures from 1 to 2 hours after the exposure but is not detectable in the serum taken 17 hours or later after the treatment. Serum x-rayed *in vitro* is devoid of stimulative action.

The lymphoid cells of rabbits and guinea pigs are so fragile as to make impossible the obtaining of counts accurate enough for experimental purposes. The serum of one species caused such rapid disintegration of the cells of another that it was impossible to determine the specificity of the reaction.

EXPLANATION OF PLATE 15.

FIG. 1. Mitotic figures found among the cells suspended in serum from x-rayed animals.

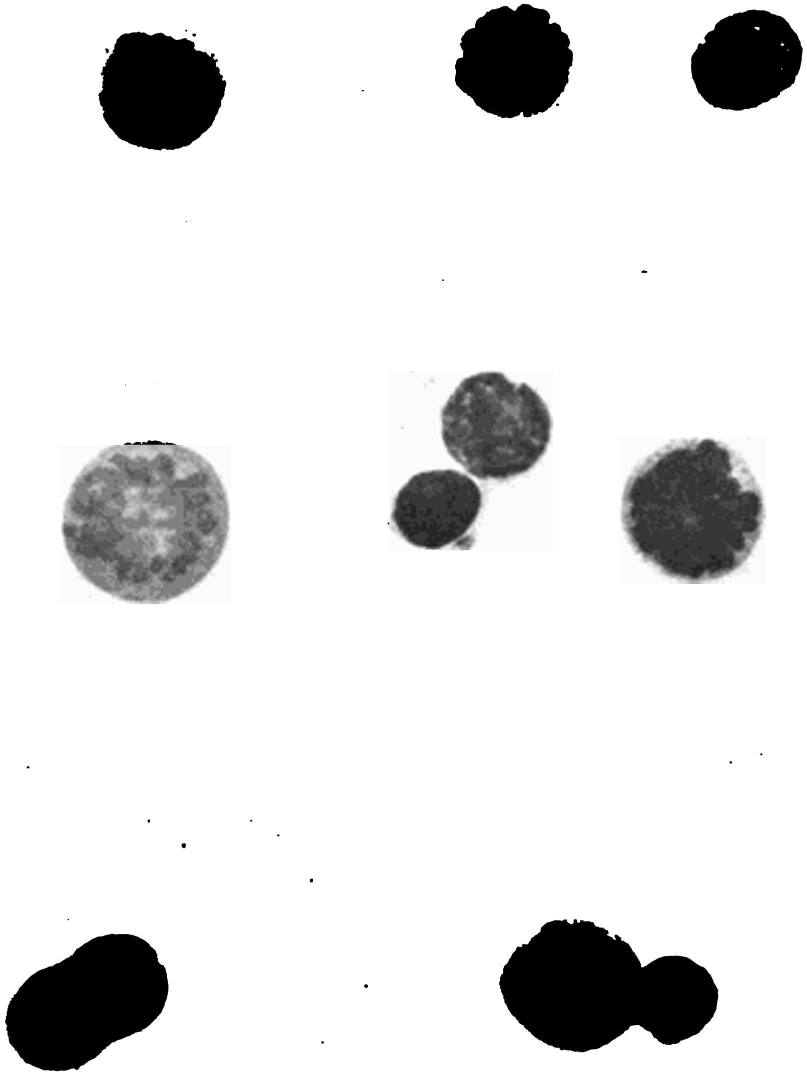


Fig. 1.

(Murphy, Liu, and Sturm: Studies on x-ray effects. IX.)

THE MEASUREMENT OF INTRAVENOUS TEMPERATURES.

BY HARRY CLARK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 16.

(Received for publication, October 20, 1921.)

The temperatures of various parts of the body, as determined with the ordinary clinical thermometer are, in general, different, and the differences are not always consistent. We cannot assume, therefore, that such measurements give a true index of the mean internal temperature of the body, which may perhaps best be identified with the temperature of the blood flowing in large blood vessels. Moreover the instrument itself is not free from objection. Its heat capacity is sufficiently great to lower appreciably for some time the temperature of tissue with which it comes in contact, and its response is too slow to follow fluctuations found with more sensitive apparatus. Although measurements made with a clinical thermometer are doubtless quite satisfactory for most purposes, it is possible that, for certain kinds of experimental work, fairly accurate determinations of blood temperatures may prove interesting. Apparatus which has been devised for this purpose by the author is described below.

The temperatures are measured by a thermoelectric method, in which one makes use of the fact that the discontinuity of electric potential at a junction of two dissimilar metals depends only on the nature of the metals and the temperature of the junction. A closed circuit of two metals contains, of course, two opposed junctions. If the second of these junctions is kept at a known constant temperature, the resultant thermoelectric force in the circuit is the difference between the potential discontinuities at the two junctions, and this is a function of the temperature of the first junction only.

This thermoelectromotive force may be used in either of two ways to measure temperature. It may be measured directly by means of galvanometer, potentiometer, and standard cell, or it may be allowed to maintain a current in the circuit which, according to Ohm's law, will be proportional to the voltage, and which may be measured by the galvanometer deflection. Each of these ways has certain advantages. In the present case the temperature range is small, being only about 5°C. above and below normal, which may be taken as 37.5°C., and the resistance of the circuit is fixed. For these reasons, and in the interest of simplicity and quickness of operation, the galvanometer deflection method was adopted. To use this method to best advantage the second thermocouple is kept constantly at 37°C. by means of a special thermostat.

The apparatus consists essentially of the unit containing the couple to be placed in the body, which is referred to as the needle unit; the portable thermostat; the cable connecting the needle unit with the thermostat; and the galvanometer with necessary switches, etc. Each of these parts is described below in detail.

The Needle Unit.

The longer unit shown in Fig. 1 is the needle unit. The couple consists of two No. 40 single-silk-covered wires, one of copper, the other of constantan, contained in the central tube of spring steel which has an external diameter of 0.7 mm. and an internal diameter of about 0.3 mm. At the needle end the wires are connected together and to the tube with solder. The technique of making this soldered joint required considerable study. It was found that a good seal could be made without allowing the solder to join the two wires together or to the tube over a length of more than 1 mm. At the other end the wires are soldered to the copper screw and the constantan center pin of the connecting plug. The steel tube lies within a hypodermic needle (external diameter 1.4 mm.), sufficient space being left between them to allow blood to flow into the glass pipette. A flow of blood shows that the needle has been properly inserted into the blood vessel and brings the needle quickly to temperature equilibrium. In the figure the tube containing the couple is shown protruding from the needle, but in practice it will be withdrawn to the best position by means of the adjusting screw. All metal parts except the electrical contacts are nickel-plated and polished. The two units shown in Fig. 1 are interchangeable, the resistance of each being 21.86 ± 0.02 ohms and the correction factor for lack of uniformity of the constantan being about 1.002.

The Flexible Cable.

Either of these units is connected to the couple contained in the thermostat by means of a flexible cable about 4 meters long. The copper and constantan conductors consist each of several No. 40 wires, each group sewed up in narrow silk ribbon. They are surrounded by a helical spring 4 mm. in diameter of piano steel wire which is covered with diagonally woven silk braid. This cable is not injured by crumpling or pulling, and is so light and flexible as not to interfere with the operator's use of the needle. The resistance is 34 ohms. The ends are provided with small receptacles, the terminals of which are of copper and constantan, to fit the connecting plugs of the needle unit and the thermostat. These receptacles also serve to connect the external metal parts of the needle unit through the steel spring of the cable to the ground plate of the thermostat.

The Thermostat.

The thermostat is shown in Figs. 2 and 3. *A* and *B* are the walls of a flask of Pyrex glass, the space between which is filled with mercury which serves both as the constant temperature bath and, by its change of volume and consequent change of level in tube *D*, as the temperature regulator. The flask is wrapped in several layers of asbestos paper through the second one of which is sewed the resistance wire for warming the bath electrically. One end of this wire is connected to a binding post on top of the thermostat and to the metal sleeve *E* which is cemented to the glass tube *D* and which carries the adjusting screw *F* with its sharp pointed platinum wire through which contact with the mercury is made. The other end of the resistance wire is connected to another binding post and to the platinum wire sealed into the flask at *L*. The flask with its wrapping sets in an ordinary pint thermos bottle, the space above the flask being filled with non-conducting material. The thermocouple consisting of the constantan wire from plug *H* and the copper wire from a copper binding post is set in wax in the glass tube *C*. The space between *B* and *C* contains mercury, above which is a packing of cloth. The copper screw of plug *H* is connected to another copper post on top of the thermostat. All posts and other metal parts having to do with the thermoelectric circuit are attached to the upper disc, *I*, of hard rubber, while the posts for heating current are set in the lower disc *K*. The metal plate *J* between these discs may be grounded. The heating current, therefore, cannot leak into the thermoelectric circuit. The cap *G* protects the screw *F* from accidental change of adjustment.

The resistance wire for the heating current has a resistance of about 28 ohms and is operated from the 110 volt lighting circuit through an external series resistance consisting of a 25 watt, 110 volt lamp. When the mercury makes contact with screw *F* the heating current is short-circuited through the mercury. With the regulator set for a temperature of 37°C., the room temperature being 20°C., the heating is *on* about 6 seconds and *off* about 10 seconds. A calculation shows that the mean fluctuation in temperature of the mercury bath could not exceed 0.02°C. under the worst conditions, but since the heat is introduced at the outer surface of the flask—the only place where heat can escape from the flask—there is no consistent flow of heat through the mercury and therefore probably only a negligible fluctuation of temperature at the middle, and this is further decreased by the presence of the mercury between *B* and *C*. The use of the mercury contact to short-circuit the heating current rather than to operate a relay has a distinct advantage in that there is no arc when the contact is broken. The currents and voltages which can be handled in this way without arc are, however, small and had to be found by preliminary experiments. This thermostat has been run almost continuously for several months and though the mercury surface is exposed to air, it is still perfectly clean and bright. Its action has been studied with a high sensitivity galvanometer and no temperature fluctuations have been found.

The Galvanometer.

A Leeds and Northrup type R galvanometer 2500-a is used, since the sensitivity is such that the desired range of temperature covers the 50 cm. scale at a distance of 1 meter, the damping is critical, and the period is only 5 seconds. It is mounted in a box backed by a grounded metal plate which is fastened to the wall. The galvanometer is packed in cotton and the box proyided with copper posts for external connection.

The external circuit from the thermostat through the switches to the galvanometer, including binding posts, wire terminals, etc., is of copper. A 50 ohm copper resistance is mounted with the switches to provide critical damping for zero readings, and to correct for slight stray thermoelectric forces in the galvanometer. The switch is mounted on a metal ground-plate. The insulated connecting wires are protected against leakage by being wrapped with copper wires fitted with terminals for connection to the ground-plates of switch, galvanometer, and thermostat.

Preliminary Tests of the Apparatus.

Calibration curves were taken with a large water bath, stirred by a motor, the temperatures being read with a microscope on a Bureau of Standards thermometer divided to 0.1°C. The curves are smooth and consistent to within 0.01°C. save for a few isolated points the worst of which are within 0.02°C. of the curve. The apparatus is therefore not more in error than the thermometer readings, probably much less. Readings accurate to 0.01°C. are obtained when the needle is immersed in water to a depth of only 3 mm., and equilibrium is reached within the normal time of damped swing of the galvanometer. Placed in the left ventricle of the heart of a rabbit, equilibrium was reached with the same degree of promptness after the flow of blood into the pipette was established. It is therefore safe to assume that the error in intravenous measurements will not be greater than 0.01°C.

EXPLANATION OF PLATE 16.

FIG. 1. The longer unit is the needle unit, the end of which is inserted into the blood vessel. The small tube inside the pipette and needle contains the two wires of the thermocouple. The shorter unit is used for taking mouth temperatures.

FIGS. 2 and 3. The thermostat which maintains the second thermocouple at a constant temperature.

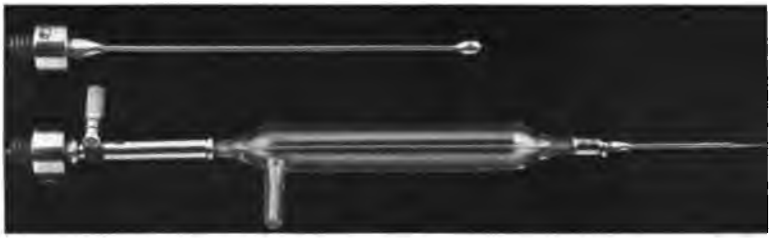
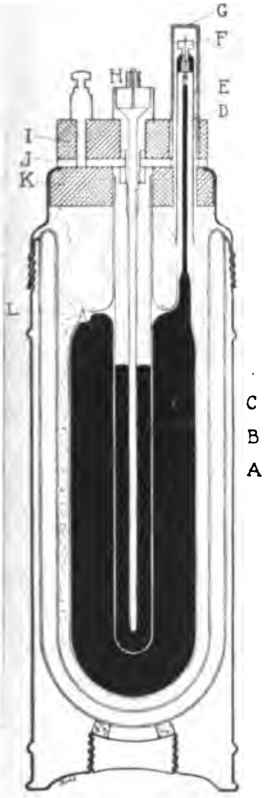


Fig. 1.



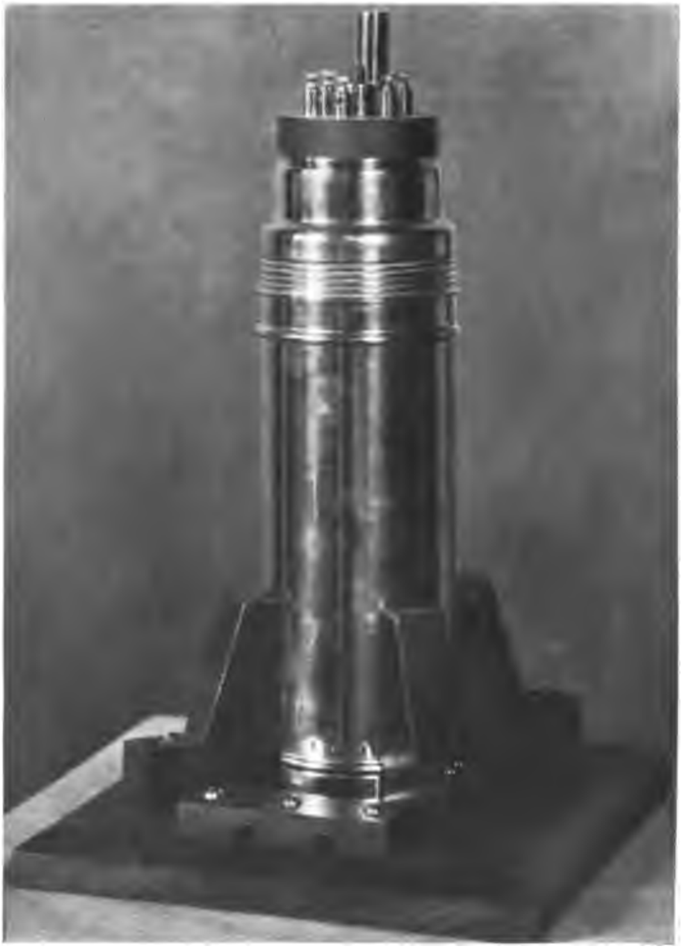


Fig. 2.

Fig. 3.

(Clark: Measurement of intravenous temperatures.)

VENEREAL SPIROCHETOSIS IN AMERICAN RABBITS.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 17 TO 20.

(Received for publication, November 10, 1921.)

Ross,[1] in 1912, in a discussion of an intracellular phase of *Treponema pallidum*, mentioned a disease of rabbits characterized by chancres, buboes, ulcers on the genitals, mouth, anus, etc., in which he reported finding cell inclusions in the mononuclear leucocytes similar to those which he had found in human syphilis. He suspected that there might be a spiral phase of the parasite of the rabbit disease, and Bayon[2] undertook the examination of the genital lesions, in which he found a spiral organism indistinguishable from *Treponema pallidum*. Similar lesions appeared in other rabbits 25 days after inoculation of the material containing the spiral organism. Bayon described the rabbit spirochete as being about one and a half times the diameter of a blood corpuscle in length, having 5 to 8 closely set, deep, rather elastic curves, and showing rotating but not lashing movements. He remarked that students of experimental syphilis in the rabbit should take note of the presence of this new species of *Treponema*.

Arzt and Kerl,[3] in 1914, reported finding in a peculiar scaly, papular, or erosive lesion, with crust, around the anogenital region of apparently normal rabbits, spiral organisms morphologically indistinguishable from *Treponema pallidum*. Of 850 rabbits examined by them, 26.9 per cent showed the same organism, and in one series of 189 adult rabbits the spirochetes occurred in 34.4 per cent. Arzt and Kerl transmitted the spirochetal affection by applying to the scarified vulva or prepuce of normal rabbits the exudate from the lesion of a rabbit showing the spontaneous disease. After several weeks incubation, the inguinal lymph nodes became somewhat enlarged, and the spirochetes appeared. Similar lesions of lips and clitoris showed the spirochete in large numbers. Intratesticular inoculations were unsuccessful, possibly because of the contaminated nature of the material, and inoculations into the scarified eyebrows of two monkeys were negative.

---

[1] Ross, E. H., An intracellular parasite developing into spirochætes, *Brit. Med. J.*, 1912, ii, 1653.

[2] Bayon, H., A new species of *Treponema* found in the genital sores of rabbits, *Brit. Med. J.*, 1913, ii, 1159.

[3] Arzt, L., and Kerl, W., Weitere Mitteilungen über Spirochätenbefunde bei Kaninchen, *Wien. klin. Woch.*, 1914, xxvii, 1053.

With a view to differentiating between this organism and *Treponema pallidum*, Arzt and Kerl[3,4] inoculated the testicle of a rabbit suffering from the disease in question with a strain of *Treponema pallidum*. The inoculation was successful, but since a syphilitic rabbit can be superinfected or doubly infected, as shown by Truffi,[5] and by Uhlenhuth and Mulzer,[6] no conclusion was drawn. No importance was attached to the Wassermann reaction, and an attempt to employ as antigen in a complement deviation test tissue from syphilitic orchitis of the rabbit failed. Examination of certain stocks in Innsbruck,[7] where no experimental work on syphilis in the rabbit had ever been done, showed the same disease to prevail.

Similar observations were made on Hamburg rabbits by Jacobsthal in 1920.[8] The lesions present were situated on the vulva and were of a squamous, erosive kind. The dark-field microscope revealed, in the lesions and in the vaginal secretion, numerous spirochetes resembling *pallidum*. Jacobsthal thought the spiral organism to be a trace thicker than *pallidum* and not to exhibit the undulatory movements so characteristic of the latter. Transmission experiments with mice and guinea pigs were negative, but lesions were reproduced in female rabbits in three instances by scarification of the vulva, after an incubation period of 3 to 6 weeks. A chancre-like lesion was noted in one animal. He proposed calling the disease *paralues cuniculi* and the organism *Spirochæta paraluis cuniculi*.[9]

Later in 1920, Schereschewsky[10] stated that the peculiar lesion of the rabbit disease is of papular and ulcerative form and is transmitted by coitus, the incubation period varying from 14 to 30 days.

In 1921 Klarenbeek[11] observed spirochetal lesions in five Dutch rabbits. Inoculations of the spirochete-containing material by scarification into the perineal

---

[4] Arzt, L., Spirochätenbefunde in Genitalveränderungen ungeimpfter Kaninchen, *Dermat. Z.*, 1920, xxix, 65.

[5] Truffi, M., Ueber die Empfänglichkeit des Kaninchens gegenüber syphilitischen Reinfektionen, *Centr. Bakt.*, *1te Abt.*, *Orig.*, 1910, liv, 337.

[6] Uhlenhuth, P., and Mulzer, P., Beiträge zur experimentellen Pathologie und Therapie der Syphilis mit besonderer Berücksichtigung der Impf-Syphilis der Kaninchen, *Arb. k. Gsndhtsamte*, 1913, xliv, 307.

[7] Arzt, L., and Kerl, W., Zur Frage der "Kaninchensyphilis," *Dermat. Woch.*, 1920, lxxi, 1047.

[8] Jacobsthal, E., Untersuchungen über eine syphilisähnliche Spontanerkrankung des Kaninchens (*Paralues cuniculi*), *Dermat. Woch.*, 1920, lxxi, 569.

[9] Arzt and Kerl[7] remind Jacobsthal that neither incubation time nor histological changes of the lesion warrant the differentiation of this disease from experimental syphilis in rabbits.

[10] Schereschewsky, J., Geschlechtlich übertragbare originäre Kaninchensyphilis und Chinin-Spirochätotropie, *Berl. klin. Woch.*, 1920, lvii, 1142.

[11] Klarenbeek, A., Recherches expérimentales avec un spirochète, se trouvant spontanément chez le lapin et ressemblant au *Treponema pallidum*, *Ann. Inst. Pasteur*, 1921, xxxv, 326.

region of normal rabbits gave rise, 11 days later, to a slightly excoriated hyperemic reaction without induration, while on the scarified dorsal skin superficial lesions with white crust appeared after 1 to 2 months incubation. The lesions showed spirochetes. Intraocular inoculations in rabbits resulted in some instances in an ulcer on the upper lid and a keratitis after 41 to 43 days; intratesticular inoculations failed to give any spirochetal orchitis.

Spontaneous Instances in America.

The facts reviewed in the foregoing paragraphs suggested the examination of American rabbits. The same condition has been found among the stock rabbits at The Rockefeller Institute. Of 50 rabbits examined in June, 1921, three females and two males were found to have lesions on the vulva, prepuce, and perineum. One (No. 3) of the female rabbits was born at The Rockefeller Institute 6 years ago, has been kept as normal breeding stock ever since, and has never been used for any experimental work.

Recently (November, 1921) six females with similar lesions have been found among twenty rabbits just purchased in Pennsylvania.

Rabbit 1.—Adult female; used for breeding purposes for several months. June 29, 1921. Scaly ulcerative lesions covered with grayish white crusts present on both sides of vulva (Fig. 38). Upon removal of the crusts with forceps, a granular, easily bleeding surface was exposed. The crust and scrapings from the exposed ulcer both revealed the presence in fairly large numbers of a spiral organism resembling *Treponema pallidum* (Figs. 1, 13, and 17). The lesions persisted without much change in extent and character up to the last examination (Sept. 26, 1921). Spirochetes were still numerous. Tissue was removed on June 29, 1921, for inoculation and histological studies. The animal was sacrificed on Sept. 27, and the lymphatic glands were examined for the spirochetes.

Rabbit 2.—Adult nursing female. June 30, 1921. Found to have small, scaly, rather dry lesions on both sides of the vulva; no ulceration. Scrapings examined by dark-field microscope showed in small numbers spirochetes resembling *pallidum*. The lesions remained unchanged up to the last examination (Oct. 13, 1921), but spirochetes were more numerous at that time. The three young rabbits remain entirely free from any lesion, although kept with the mother during a period of 3 months.

Rabbit 3.—Adult female; born at The Rockefeller Institute in 1915 and kept as breeding stock since. June 30, 1921. Scaly, papular elevation or thickening of the vulval region on both sides (Fig. 39), covered by thin grayish crusts over the center of the hyperemic lesions. Examination for spirochetes revealed one fine type in enormous numbers (Figs. 2 to 4, 14, 15, and 18 to 20) and another decidedly coarser type in smaller numbers (Figs. 5, 6, and 27 to 29). Tissue was excised on June 30, and July 15, 1921, for section and for inoculation. On July 20, 1921, there was no perceptible change in the lesion, and numerous spirochetes were still present (Figs. 9 to 11). This animal was later treated with salvarsan (see below).

Rabbit 4.—Normal adult male; recently purchased.[7] July 6, 1921. Presented small congested and quite swollen but not indurated lesions of the prepuce (Fig. 40), covered with thin crusts; the lesions bled easily when the crusts were removed. The dark-field microscope showed numerous spirochetes similar to those found in the other animals (Figs. 7, 8, 16, and 21 to 24). On July 20, 1921, there were still spirochete-containing lesions. Toward the end of September scaly papular lesions were noticed on nose and lips (Fig. 42), eyelid (Fig. 43), and paws (Fig. 44), and the spirochetes were found to be present there also.

Rabbit 5.—Normal adult male; recently purchased. Sept. 10, 1921. Prepuce swollen and covered with grayish thin scales, under which the tissue was granular, soft, somewhat edematous, and congested (Fig. 41). Numerous spirochetes present. Lesion excised for histological examination.

Spirochetes.

The type of spirochete existing in the lesions of the five rabbits just described closely resembles *Treponema pallidum*. The measurements are: length 10 to 16 μ; width 0.25 μ. The spiral amplitude, in regular portions, is about 1 to 1.2 μ, and the spiral depth 0.6 to 1 μ; hence the number of spirals in an individual measuring 10 μ may be about 9 or 10. The last spirals near the extremities are often shallower than those of the middle portion or *vice versa*, and the ends are sharply drawn. A delicate terminal filament has been seen at one, and sometimes at both ends. The shortest specimens measured 7 μ, with 6 spirals, and the longest about 30 μ, with 25 to 28 spirals (Fig. 4). The average is about 12 to 14 μ (Figs. 3, 5, and 6), the organism being slightly longer than the *pallidum* in human lesions. In experimentally inoculated rabbits specimens of *pallidum* as long as 24 μ may be encountered, but the single length is usually 10 to 12 μ (Figs. 12 and 32 to 34). As in the case of *pallidum* (Figs. 35 to 37), specimens with stretched spirals appear in stained preparations (Figs. 25 and 26).

In the lesion of Rabbit 3 there were present two types of spirochete, one the usual fine type and the other decidedly coarser than the average.[8] The morphology of the coarser variety suggests that of

Treponema calligyrum found by the writer in the human condyloma,[12] but it is somewhat longer than *calligyrum* (14 to 18 μ, width 0.35 μ). The spiral amplitude is 1.2 to 1.75 μ and the spiral depth 0.6 to 1.5 μ (Figs. 6 and 27 to 29). This form is perhaps a trace thinner and longer than the *calligyrum*, or medium type smegma treponema of the normal human genitalia. The organism rotates actively, with occasional bending at the middle, but no vibratory or lashing movements have been observed.

The rabbit treponema forms entangled masses of long threads, 30 μ at least (Figs. 4 and 11), a phenomenon I do not recall having seen in the case of *Treponema pallidum*. The agglomeration of organisms in a striking stellate mass of several to a dozen individuals is frequently observed (Fig. 10). A similar behavior has been observed in young, actively motile, pure cultures of *Treponema pallidum*. Both *pallidum* and the rabbit treponema sometimes form a ring by touching one end with the other while actively rotating, and occasionally they make a knot of themselves. In a film preparation from the lesions of Rabbit 3, stained by Fontana's method, occasional wavy, but not spiral forms were seen; perhaps they were degenerated or involution forms (Figs. 30 and 31).

The staining properties of these rabbit spirochetes are the same as those of *Treponema pallidum*. Both can be readily stained by ordinary basic aniline dyes when fixed in a buffered formaldehyde solution, as shown by Miss Tilden.[13] Jacobsthal proposed for the organism the name *Spirochæta paraluis cuniculi*, but in view of its close resemblance to *Treponema pallidum* and *Treponema pertenue* it may perhaps be more appropriate to use the simpler name *Treponema cuniculi*.

Examinations of smegma and mucous secretions on the genitalia of many normal male and female rabbits failed to reveal any spirochetal organisms, which circumstance does not of course exclude the possibility that spirochetes are occasionally present in such materials.

---

[12] Noguchi, H., Cultivation of *Treponema calligyrum* (new species) from condylomata of man, *J. Exp Med.*, 1913, xvii, 89.

[13] Noguchi, H., A note on the venereal spirochetosis of rabbits. A new technic for staining *Treponema pallidum*, *J. Am. Med. Assn.*, 1921, lxxvii, 2052.

Histology of the Lesions.

Excised tissue from the undisturbed spontaneous lesion on the prepuce of Rabbit 5 (Fig. 41) was fixed in Zenker's fluid and sections were stained with hematoxylin-eosin and eosin-methylene blue. The epidermis was thickened and the papillæ were large and prominent; the infiltration was spotty and mainly confined to the interpapillary layers. The new cells were mainly mononuclear, partly eosinophils, and few or no polymorphonuclears were present. In general features the lesion suggests a condyloma. Although certain blood vessels were surrounded by mononuclear cells, no definite endothelial proliferation was apparent (Fig. 48). The lesion as described may be taken as the pure type before secondary bacterial infection has come in to induce acute inflammation and mar the picture. Once ulceration has taken place, as in the vulval lesions of Rabbits 1 and 3 (Figs. 38 and 39), the interpapillary infiltration of mononuclear cells is accompanied by numerous polymorphonuclear leucocytes, which invade also the stratified layers of the thickened epidermis. Minute hemorrhages and necrotic foci are then occasionally met with.

For comparison with these lesions, a scrotal chancre was induced with the Nichols strain of *Treponema pallidum* long adapted to the rabbit (since 1912). The chancre on the 28th day was indurated and stood out as an oval mass from the surrounding loose connective tissue (Fig. 47); in histology it differed widely from the genital lesions induced by the rabbit spirochete. The corium was highly infiltrated with mononuclear cells, some showing eccentric nuclei and checker-board chromatin. Polymorphonuclear leucocytes were absent. But the striking difference was in the blood vessels, which were surrounded by the new cells (Fig. 49).

Repeated attempts to stain the spiral organism in the tissue by means of silver impregnation have been only partially successful. The organisms near the surface of the lesion took the stain. By careful teasing of the deeper layers and dark-field examination, spirochetes have been found to be present in all parts of the lesions.

Transmission Experiments.

First Passages.—Sixteen rabbits and four monkeys[14] (*Macacus rhesus*) were inoculated with the material containing the spirochetes, usually on the scarified prepuce or vulva. In rabbits some intratesticular, intracorneal, and intraocular inoculations were made; in monkeys the scarified eyebrows, as well as prepuce or vulva, were smeared with the scraping of the rabbit lesion. Each of the original rabbits was paired in a separate cage with a normal rabbit of opposite sex for the purpose of sexual transmission.

Reproduction of the disease on the vulva or prepuce respectively was obtained in all five of the female and in eight of the eleven male rabbits inoculated (Table I). One strain (Rabbit 3) produced in two male rabbits a superficial scaly, papular lesion on the scrotal skin at the site of testicular inoculation (Fig. 45), and in one of them a lesion developed also on the prepuce. The results of intratesticular inoculation were in general unsatisfactory. In most cases, owing to the impure nature of the material, a more or less acute suppurative orchitis ensued, and no spirochetes were present in these lesions. Negative results also followed intraocular and intracorneal inoculations and the application of the material to the scarified surface of the cornea. The lymph glands were not noticeably enlarged either in the original rabbits or in those inoculated. The inoculation of emulsions of popliteal and inguinal lymph glands from ten rabbits infected with *Treponema cuniculi* into both testicles of four normal male rabbits did not produce any orchitis within 3 months. On the other hand, similar emulsions from rabbits infected with *Treponema pallidum* gave rise to typical orchitis within 1 month.

---

[14] Four additional monkeys were later inoculated with different strains of *Treponema cuniculi*, but none showed any lesion during a period of observation of 3 months. In order to determine whether the adaptation to rabbits of the human strain of *Treponema pallidum* had rendered it avirulent for monkeys, two monkeys were inoculated with the Nichols strain (1912). Both presented typical lesions within 1 month. Two monkeys inoculated from a case of human chancre showed typical lesions within 2 months. The pathogenicity of *Treponema cuniculi* appears, therefore, to be different from that of *Treponema pallidum* of human syphilis.

TABLE I.

Transmission Experiments.

Animal No.	Sex.	Source of inoculated material.	Site of inoculation.	Date and mode of inoculation.	Results.	Period of incubation.	Remarks.
Rabbits.						*days*	
6	Female.	Rabbit 1	Vulval mucosa.	June 30, 1921. Scarification and smearing.	Scaly lesion at site of inoculation first noticed Sept. 26, 1921.	88	
7	"	" 1	" "	" "	Definite lesion Sept. 13, 1921.	75	
8	Male.	" 1	Prepuce.	" "	Ulcer with thin crust on prepuce Aug. 10, 1921.	41	
9	"	" 1	"	" "	No lesion after 90 days.		
10	"	" 1	"	" "	Definite scaly lesion around anus Sept. 26, 1921.	88	
			Right cornea.	" "	No lesion after 90 days.		
			" testicle.	June 30, 1921. 1 drop intratesticularly.	No orchitis after 90 days.		
11	"	" 1	Prepuce.	June 30, 1921. Scarification.	Definite lesion Sept. 26, 1921.	88	
			Right cornea.	" "	No lesion after 90 days.		

11	Male.	Rabbit 1	Right testicle.	June 30, 1921. 1 drop intratesticularly.	Abscess formation and induration lasting for 1 mo. No spirochetes found in testicular puncture.		
Macacus rhesus. 1	"	" 1	Prepuce.	June 30, 1921. Scarification.	No lesion after 80 days.		Reinoculated with material from Rabbit 4, Sept. 19, 1921.
			Left eyebrow.	" "	" "		
2	"	" 1	Prepuce.	" "	" "		
			Left eyebrow.	" "	" "		" "
Rabbits. 12	Female.	" 2	Vulva.	July 1, 1921. Scarification.	Scaly lesion Sept. 13, 1921, since spread.	75	
13	"	" 2	"	" "	Small lesion July 20, 1921, now slightly increased.	20	
14	Male.	" 2	Prepuce.	" "	Definite lesion Aug. 17, 1921.	47	
			Cornea.	" "	None.		
			Right testicle.	July 1, 1921. Intratesticular.	Abscess formation in 7 days, extended to left testicle.		

TABLE I—*Concluded.*

Animal No.	Sex.	Source of inoculated material.	Site of inoculation.	Date and mode of inoculation.	Results.	Period of incubation.	Remarks.
Rabbits.						*days*	
15	Male.	Rabbit 2	Prepuce.	July 1, 1921. Scarification.	Definite lesion Sept. 26, 1921.	88	
			Cornea.	" "	None.		
			Right testicle.	July 1, 1921. Intratesticular.	Suppurative lesions after 1 wk., reremained firm for 40 days.		
Macacus rhesus.							
3	"	" 2	Prepuce.	July 1, 1921. Scarification.	None.		
			Right eyebrow.	" "	"		
4	Female.	" 2	Vulva.	" "	"		
			Eyebrow.	" "	"		
Rabbits.							
16	"	" 3	Vulva.	July 2, 1921. Scarification.	Definite lesion Aug. 16, 1921.	45	
17	Male.	" 3	Prepuce.	" "	Definite lesion Aug. 17, 1921.	46	
			Both testicles.	July 2, 1921. Intratesticular.	Scab at lower part of left scrotum Aug. 17, 1921. Lesion slightly indurated and many spirochetes found.	46	
18	"	" 3	Prepuce.	July 2, 1921. Scarification.	No lesion developed.		

18	Male.	Rabbit 3	Right testicle.	July 2, 1921. Intratesticular.	Abscess formation. Scaly lesions on left scrotum Aug. 20, 1921. Spirochetes+.	49	Photographed (Fig. 45) Sept. 13, 1921 (73 days).
19	"	" 3	Prepuce.	July 5, 1921. Scarification.	Definite lesion Sept. 13, 1921.	70	
			Left testicle.	July 5, 1921. Intratesticular.	No lesion developed.		
Macacus rhesus.							
3	"	" 3	" eyebrow.	July 2, 1921. Scarification.	" " "		Right eyebrow had been inoculated from Rabbit 3 on July 1, 1921.
Rabbits.							
20	"	" 4	Prepuce.	July 5, 1921. Scarification.	Definite lesion Aug. 10, 1921.	36	
			Cornea.	July 2, 1921. Intracorneally with syringe.	Opacity still persists after 85 days.		
			Both testicles.	July 2, 1921. Intratesticular.	Two small nodules on left testicle (no spirochetes).		
21	"	" 4	Prepuce.	July 5, 1921. Scarification.			Died of septicemia in 8 days.
			Eye.	July 5, 1921. Intraocular (anterior chamber).			
			Both testicles.	July, 5, 1921. Intratesticular.			
Average..........						60⅓	

The period of incubation varied from 20 to 88 days, the average being 60½ days.

Subsequent Passages.—All five strains are being maintained in rabbits. The strain from Rabbit 1 has now passed through four transfers: first, June 30, 1921, positive September 13 (75 days); second, September 27, positive October 17 (20 days); third, October 17, positive October 22 (5 days); fourth, October 24, positive November 10 (17 days).

Inoculations of monkeys have remained up to the present (4 months) without result. One of the monkeys was inoculated with two different strains, on right and left eyebrow respectively. Two other monkeys have recently been reinoculated in the eyebrow, and also into the hypogastric region, with a hypodermic needle, the infective material being deposited intradermally.

Sexual Transmission.—Rabbit 1 was kept in the same cage with three males successively (July 12 to September 26), Rabbit 2 with two males, and Rabbit 3 with one male (July 12 to October 15). Rabbit 22, 3 months after having been placed with Rabbit 3, showed a small papular lesion on the prepuce in which spirochetes were numerous.

Attempts to Produce Chancre on the Scrotum.

Treponema pallidum from human syphilis is capable of producing chancre-like lesions on the scrotum of rabbits when a small piece of *pallidum*-containing tissue is subcutaneously introduced (Tomasczewski,[15] Uhlenhuth and Mulzer,[6] Brown and Pearce,[16] and others). To determine whether or not the rabbit spirochete is capable of producing a similar lesion in rabbits, portions of the erosive, spirochete-containing lesion from the vulvolabial region of Rabbit 3 were introduced into the subcutaneous pocket of each of the scrota of two adult male rabbits, two other adult males being simultaneously inoculated with fragments of freshly excised scrotal chancre from a syphilitic rabbit (Nichols strain).

---

[15] Tomasczewski, Ueber eine einfache Methode, bei Kaninchen Primäraffekte zu erzeugen, *Deutsch. med. Woch.*, 1910, xxxvi, 1025.

[16] Brown, W. H., and Pearce, L., Experimental syphilis in the rabbit. II. Primary infection in the scrotum. Part 1. Reaction to infection, *J. Exp. Med.*, 1920, xxxi, 709.

One of the rabbits (No. 23) inoculated with the rabbit spirochete showed at one site of insertion after 27 days a small crust under which the surface was soft, pale, and granular, but there was no marked induration. Spirochetes were not found in the lesion until 45 days after inoculation. The other site remained small and reddish, with a trace of infiltration, but no spirochetes were found. The other rabbit (No. 24) showed slight infiltration at one site of insertion (Fig. 46), but no definite, chancre-like induration developed during 2 months observation. Brownish scaly patches rich in spirochetes appeared on the right scrotum after 45 days.

Of the two syphilitic controls, Rabbit 25 developed within 3 weeks at the site of insertion on the right scrotum a typical chancre which attained a diameter of 2 cm. in 28 days (Fig. 47). The second control (Rabbit 26) had not developed a chancre 75 days after inoculation. Two rabbits inoculated with chancre material from Rabbit 25 developed typical chancres within 3 weeks, while simultaneous inoculations from the slightly indurated (*cuniculi*) lesion of Rabbit 23 were unsuccessful.

It is possible that a chancre may yet be produced with the rabbit spirochete by inoculation of a large number of animals. The number of spirochetes in the *pallidum* chancre of rabbits is never as large as that found in the *cuniculi* lesions in rabbits.

For the purpose of comparison, *pallidum*-containing material was applied to the scarified prepuce or vulva of rabbits and gave rise to chancre-like nodules. The appearance of these lesions, however, was entirely different from that of the lesions produced by *Treponema cuniculi*.

Typical *cuniculi* lesions may be produced on the scrotal skin or prepuce of rabbits having active *pallidum* lesions, and the reverse is also true. The infections appear to follow independent courses.

Wassermann Reaction.

The sera of the eleven rabbits with spontaneous lesions, and of thirty-three experimentally inoculated from them, have been tested for the Wassermann reaction. The acetone-insoluble lipoids served as antigen, and the inactivated sera were used in quantities of 0.1 and 0.2 cc. Twenty-four of the animals had active, spirochete-containing lesions of the genital region at the time of test; twenty had recently been inoculated and had not developed any lesions. The results of the tests were uniformly negative.

The sera of four rabbits having active scrotal chancres (Nichols strain of *Treponema pallidum*) were simultaneously tested; three gave a strongly positive (++++), and the other a partial (+) reaction. Twelve rabbits which had developed chancres 2 months after inoculation on vulva or prepuce with *pallidum* gave strongly positive (++++) reactions.

Thus far positive Wassermann reactions have been obtained only with the sera of rabbits having active syphilitic lesions. Apparently no complement-fixing substance, such as exists in the blood of syphilitic human beings or experimentally infected syphilitic rabbits, was present in the blood of animals which had been infected with the rabbit treponema for periods varying from 1 or 2 weeks to many months. Whether or not the fixing substance will eventually appear in the sera of these rabbits remains to be seen.

Effect of Salvarsan.

Rabbit 3 was given intravenously on August 17, 1921, 0.02 gm. of salvarsan per kilo of body weight. Rabbit 27, having a chancre of thumb size on each scrotum, was similarly treated at the same time to serve as control.

Rabbit 3.—Aug. 17, 1921. Showed papuloedematous, partially eroded, moist lesion, extending over the entire vulval region, including the labial mucosa and adjacent skin; perineum also scaly, congested, and swollen. Dark-field examination revealed spirochetes in large numbers. 5 p.m. 4.4 cc. of a 1:10 dilution of slightly alkaline salvarsan solution was intravenously administered (0.02 gm. per 1,000 gm. of body weight). Aug. 18, 5 p.m. Considerable reduction in swelling, less irritation, apparent drying up of lesion; no spirochetes found. Aug. 20. Lesion near hair line of vulva still somewhat moist and crusted, inferior portion practically healed; anal lesion still present. Spirochetes were no longer found in any of the suspicious spots. Aug. 26. Only small scabs present. Sept. 13. Slight scaliness at hair line of vulva; no spirochetes.

Rabbit 27.—Aug. 17, 1921. Chancre (human strain isolated by Nichols in 1912, since maintained in rabbits) on each scrotum, 2 cm. in diameter; cartilaginous induration, with small umbilicated center covered by dark dry crust. Many spirochetes present. 5 p.m. 5 cc. of a 1:10 dilution of slightly alkaline salvarsan (0.02 gm. per 1,000 gm. of body weight) intravenously administered. Aug. 18. Lesions softer, smaller; no spirochetes. Aug. 20. Lesions soft. Aug. 23. Lesions have almost disappeared. Sept. 13. No trace of lesions found.

The experiment indicates that the *cuniculi* lesions and the experimental scrotal chancre caused by *Treponema pallidum* from human syphilis are similarly influenced by salvarsan. In both instances the spirochetes disappeared within 24 hours after the administration of the drug, and the lesions themselves within 9 days. No recrudescence has occurred.

DISCUSSION AND SUMMARY.

Of 50 rabbits, otherwise regarded as normal, three adult females and two adult males (10 per cent) have been found to have in their genitoperineal region certain papulosquamous, often ulcerating, lesions. A recently purchased group of twenty rabbits contained six females (30 per cent) with similar lesions. This condition runs a chronic course and is characterized by the presence of a spiral organism closely resembling *Treponema pallidum.*

The rabbit spirochete has the same morphological features as *Treponema pallidum*; it is possibly a trifle thicker and longer than the average *pallidum.* Long specimens measuring 30 μ are frequently encountered, and they show a tendency to form loosely entangled knots. A stellate arrangement of several organisms in a mass is frequently observed.

In the lesion of one rabbit there were two types of spirochete, one of the variety just described, the other a somewhat coarser organism, closely resembling *Treponema calligyrum* found in a human condyloma, but a trifle thinner and longer. This organism is perhaps merely a variant type of the rabbit spirochete.

The histological reactions are similar to, but considerably less cellular, than those occurring in typical primary syphilitic lesions. There is a marked hyperkeratosis and interpapillary infiltration not observed in scrotal chancre.

The disease is transmissible to normal rabbits, in which the usual papular lesions can be readily reproduced in the genitoperineal region. In the first passages the incubation period varied from 20 to

88 days; subsequently one of the strains produced a lesion in 20 days on the second, and in 5 days on the third passage. No typical orchitis or keratitis was produced in the rabbits of the present series, although in one of the original rabbits (No. 4) scaly, papular lesions have developed on the nose, lips, eyelid, and paws. Monkeys (*Macacus rhesus*) failed to show any lesions within a period of 4 months after inoculation.

In one instance transmission was accomplished through the mating of an infected female with a normal male.

The Wassermann reaction was uniformly negative in the five rabbits with spontaneous lesions and in eighteen rabbits experimentally infected.

Salvarsan had the same therapeutic effect on the lesions produced by the rabbit spirochete as on the experimental *pallidum* lesion of the rabbit.

The organism belongs to the genus *Treponema,* and may be designated *Treponema cuniculi.*

EXPLANATION OF PLATES.

PLATE 17.

Dark-field photographs. Magnification × 1,000.

FIG. 1. *Treponema cuniculi* from Rabbit 1.

FIGS. 2 to 5. *Treponema cuniculi* from Rabbit 3. Fig. 4 shows one of the very long specimens.

FIG. 6. The coarser type of treponema from Rabbit 3.

FIGS. 7 and 8. *Treponema cuniculi* from Rabbit 4.

FIGS. 9 to 11. *Treponema cuniculi* from Rabbit 3. Fig. 11 shows an entangled mass of the long forms. Fig. 10 illustrates the agglomeration of organisms in stellate form which is frequently seen.

FIG. 12. *Treponema pallidum* from experimental syphilitic orchitis in the rabbit.

PLATE 18.

Magnification × 1,000.

FIG. 13. Film preparation of exudate from Rabbit 1, stained with Giemsa solution.

FIGS. 14 and 15. Film preparation of exudate from Rabbit 3, stained with Giemsa solution.

FIG. 16. Film preparation of exudate from Rabbit 4; Giemsa stain.

FIG. 17. Film preparation from Rabbit 1, stained by Fontana's method.

FIGS. 18 to 20. Film preparation from Rabbit 3, stained by Fontana's method.

FIGS. 21 to 24. Film preparation from Rabbit 4, stained by Fontana's method.

FIGS. 25 and 26. Irregular forms of *Treponema cuniculi* from Rabbit 3, stained by Fontana's method.

FIGS. 27 to 29. Spirochetes resembling *Treponema calligyrum* from Rabbit 3, stained by Fontana's method.

FIGS. 30 and 31. Non-spiral forms from Rabbit 3.

FIGS. 32 and 33. *Treponema pallidum* in rabbit orchitis, stained by Fontana's method.

FIG. 34. *Treponema pallidum* in rabbit orchitis. Mordant Gentian violet.

FIGS. 35 to 37. Irregular forms of *Treponema pallidum* from experimental scrotal chancre, stained by Fontana's method.

PLATE 19.

FIGS. 38 to 41. Typical lesions (from Rabbits 1, 3, 4, and 5 respectively) on the rabbit genitalia.

FIG. 42. Lesions in Rabbit 4 on the nose and left upper lip.

FIG. 43. The same, on the left eyelid.

FIG. 44. The same, on the left paw near the joints of the nails.

FIG. 45. An erosive lesion at the site of intratesticular inoculation in Rabbit 18 (transfer from No. 3) after an incubation period of 49 days. There was only a slight induration, but numerous spirochetes were present.

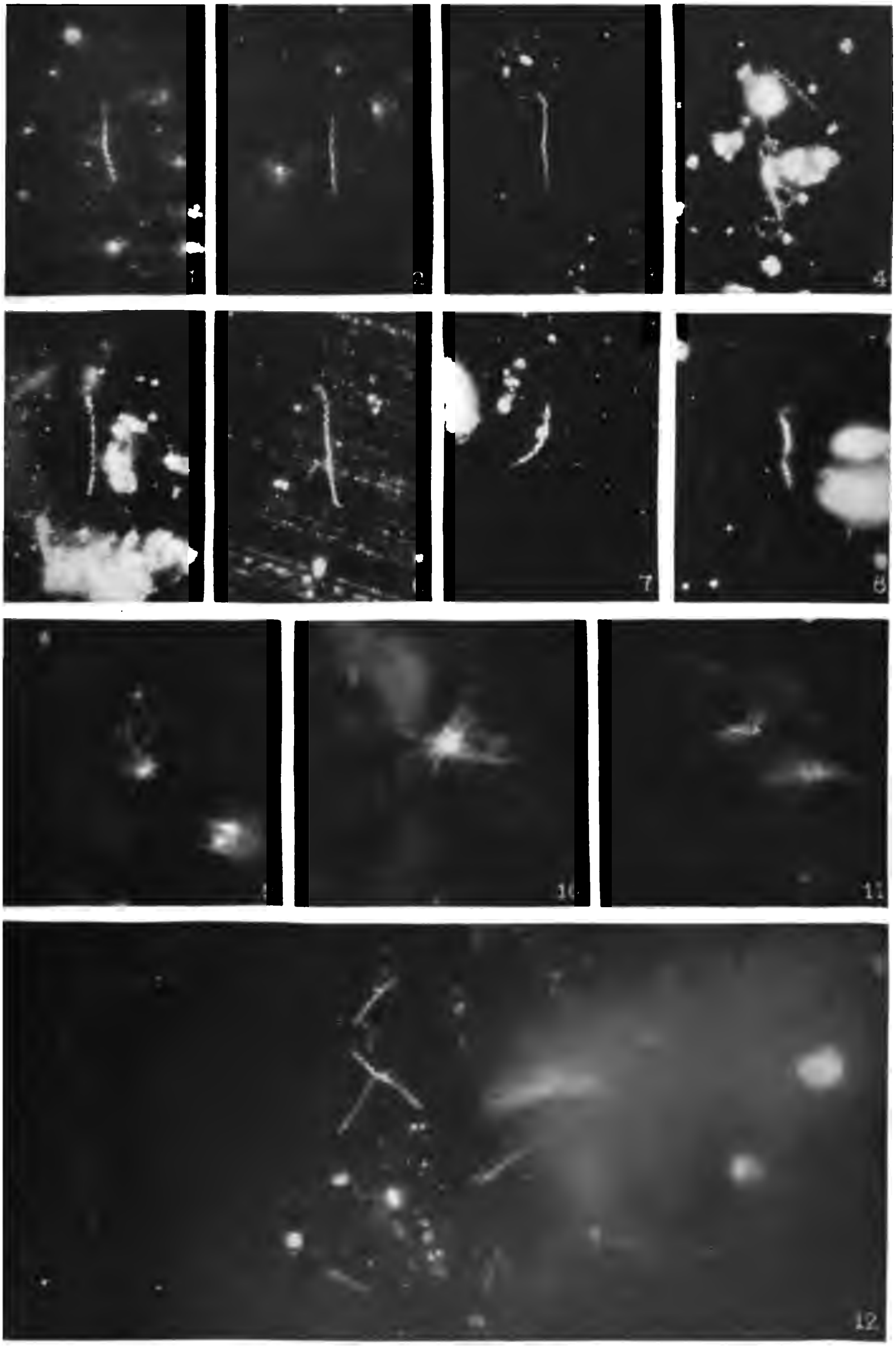
FIG. 46. The healing scar in Rabbit 24 at the site of pocket insertion of the infective material in the unsuccessful attempt to produce chancre (photograph taken 28 days after inoculation). *Treponema cuniculi* found after 73 days.

FIG. 47. A typical chancre, for comparison, on the scrotum of Rabbit 25, inoculated with the Nichols strain of *Treponema pallidum*. Note the cartilaginous, well circumscribed, umbilicated chancre (photographed 28 days after insertion).

PLATE 20.

FIG. 48. Histological changes of the lesion on the prepuce of Rabbit 5. Zenker fixation, stained by hematoxylin-eosin. Showing the marked hyperkeratosis and considerable mononuclear infiltration of the interpapillary spaces. × 75.

FIG. 49. Section of chancre on scrotal skin of Rabbit 25 (Nichols strain) 28 days after inoculation, showing typical interstitial and perivascular infiltration of mononuclear cells, and almost normal epidermis. The same fixation and staining as Fig. 48, shown for comparison. × 75.

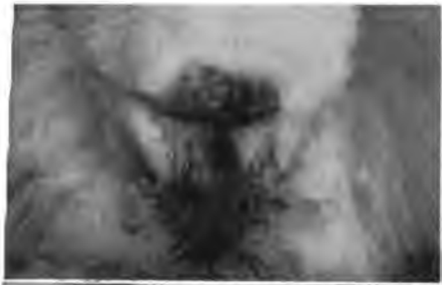


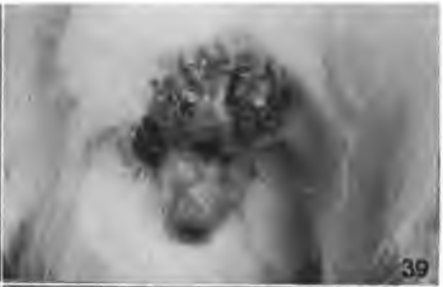
(Noguchi: Venereal spirochetosis in American rabbits.)

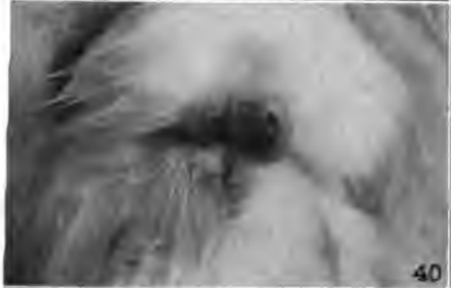
TABLE I—*Concluded.*

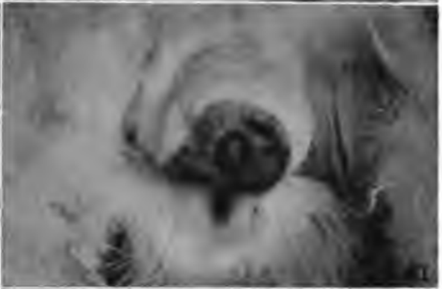
Animal No.	Sex.	Source of inoculated material.	Site of inoculation.	Date and mode of inoculation.	Results.	Period of incubation.	Remarks.
Rabbits.						*days*	
15	Male.	Rabbit 2	Prepuce.	July 1, 1921. Scarification.	Definite lesion Sept. 26, 1921.	88	
			Cornea.	" "	None.		
			Right testicle.	July 1, 1921. Intratesticular.	Suppurative lesions after 1 wk., reremained firm for 40 days.		
Macacus rhesus.							
3	"	" 2	Prepuce.	July 1, 1921. Scarification.	None.		
			Right eyebrow.	" "	"		
4	Female.	" 2	Vulva.	" "	"		
			Eyebrow.	" "	"		
Rabbits.							
16	"	" 3	Vulva.	July 2, 1921. Scarification.	Definite lesion Aug. 16, 1921.	45	
17	Male.	" 3	Prepuce.	" "	Definite lesion Aug. 17, 1921.	46	
			Both testicles.	July 2, 1921. Intratesticular.	Scab at lower part of left scrotum Aug. 17, 1921. Lesion slightly indurated and many spirochetes found.	46	
18	"	" 3	Prepuce.	July 2, 1921. Scarification.	No lesion developed.		

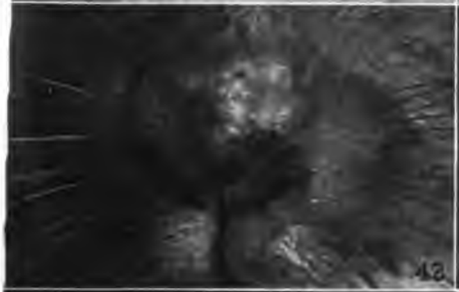
18	Male.	Rabbit 3	Right testicle.	July 2, 1921. Intratesticular.	Abscess formation. Scaly lesions on left scrotum Aug. 20, 1921. Spirochetes+.	49	Photographed (Fig. 45) Sept. 13, 1921 (73 days).
19	"	" 3	Prepuce.	July 5, 1921. Scarification.	Definite lesion Sept. 13, 1921.	70	
			Left testicle.	July 5, 1921. Intratesticular.	No lesion developed.		
Macacus rhesus.							
3	"	" 3	" eyebrow.	July 2, 1921. Scarification.	" " "		Right eyebrow had been inoculated from Rabbit 3 on July 1, 1921.
Rabbits.							
20	"	" 4	Prepuce.	July 5, 1921. Scarification.	Definite lesion Aug. 10, 1921.	36	
			Cornea.	July 2, 1921. Intracorneally with syringe.	Opacity still persists after 85 days.		
			Both testicles.	July 2, 1921. Intratesticular.	Two small nodules on left testicle (no spirochetes).		
21	"	" 4	Prepuce.	July 5, 1921. Scarification.			Died of septicemia in 8 days.
			Eye.	July 5, 1921. Intraocular (anterior chamber).			
			Both testicles.	July, 5, 1921. Intratesticular.			
Average..						60⅓	

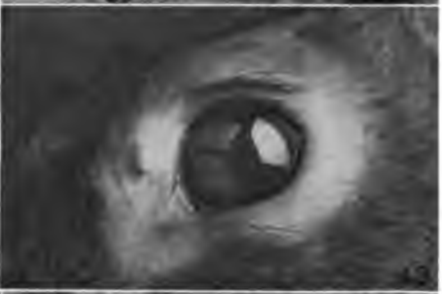


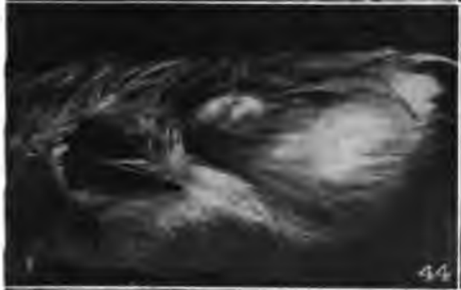

39

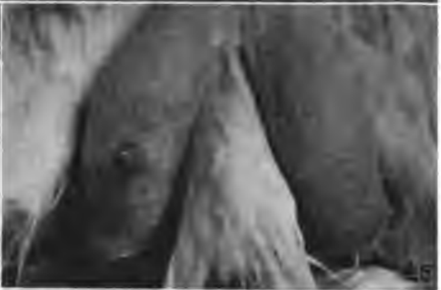

40

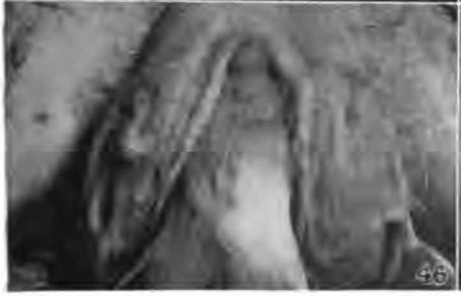

41

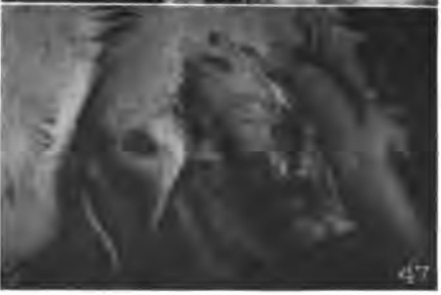

42


43

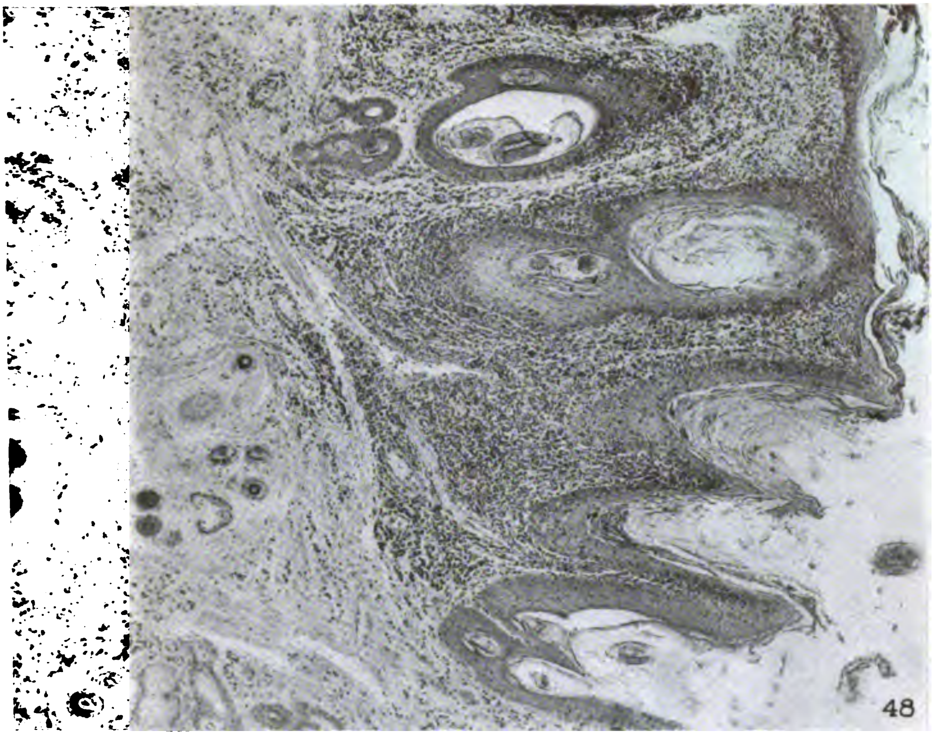

44


45

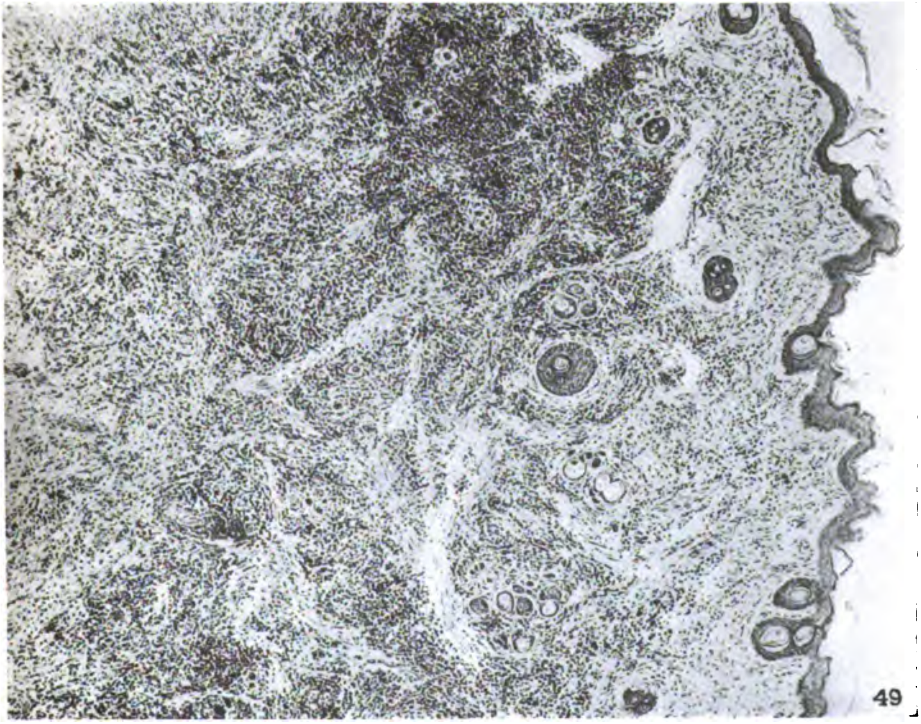

46


47

(Noguchi: Venereal spirochetosis in American rabbits.)



48



49

(Noguchi: Venereal spirochetosis in American rabbits.)

3 2044 103 071